Soil legacy and fungal community responses to *Cytisus scoparius* invasion

A thesis submitted in partial fulfilment of the requirements for the degree of Doctor of Philosophy in Biology

2020

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General abstract

The goal of my thesis was to study the effects of soil under various levels of invasive *Cytisus scoparius* (Scotch broom) and then examine whether the unique soil legacy of *C. scoparius* was contingent on how *C. scoparius* shaped soil fungal communities.

I began my research by studying the effect of the soil legacy of *C. scoparius* in a controlled environment (via a greenhouse experiment; Chapter 2). Knowing the effect of the soil legacy of *C. scoparius* under regulated conditions, I then undertook a field survey (via a natural experiment; Chapter 3), in which I systematically recorded changes in fungal community composition across a natural density gradient of *C. scoparius* invasion. I subsequently investigated whether the environmental DNA (eDNA) metabarcoding techniques I applied throughout my natural survey could be optimised for future researchers (via a methodological experiment; Chapter 4). Lastly, I analysed how different fungal communities found near *C. scoparius* may underlie the results of my greenhouse experiment (via mixed-effect modelling; Chapter 5).

In Chapter 2, I found contrary to my hypothesis that the effects of soil extracted under various levels of *C. scoparius* invasion favoured the growth of native New Zealand plants over its own taxonomic family in a controlled greenhouse environment. Given that the predominantly positive soil legacy of *C. scoparius* could only be partly attributed to soil chemical traits, microbial effects likely played an underlying role in the invasion success of *C. scoparius.* In Chapter 3, I found that fungal diversity in soil under *C. scoparius* was unexpectedly higher than in grassland uninvaded by *C. scoparius,* and that *C. scoparius* invasion resulted in increased homogenisation of certain fungal groups within the overall soil fungal community. My results suggested that coalescence between previously separated fungal communities may have occurred due to *C. scoparius* invasion. Apart from *C. scoparius* having a definite effect on soil fungal communities, it is possible that the soil fungal communities themselves might contribute to the shrub's invasiveness, which I further tested in a field-experiment (Appendix E). In Chapter 4, I present the pitfalls and benefits of eDNA pooling, identifying a fungal taxon-wide bias in the proportional abundance of fungi in pooled eDNA samples. I demonstrate how rarer fungi remain increasingly unaccounted for with increased degrees of pooling, yet also show how pooling may benefit researchers who wish to study the larger-scale effect of environmental drivers (e.g., anthropogenic effects, invasive species impacts). In Chapter 5, I show how increased arbuscular mycorrhizal richness found in more homogenised soil communities (studied in Chapter 1) were partly responsible for the generally positive soil legacy of *C. scoparius*, especially for exotic Fabaceae which can probably benefit more from arbuscular mycorrhizal fungi-facilitated P enrichment due to their ability to fix N.

By demonstrating how changes in fungal communities caused by an invasive N-fixing plant may impact plant growth and nutrient acquisition, the results of my thesis highlight the importance of incorporating fungal community composition in soil legacy studies. Although biodiversity losses of plants and other organisms following invasion are common, I show how soil fungal communities may be considered an exception to the rule. I highlight the importance of systematic sample processing and encourage the use of eDNA metabarcoding techniques to better understand how changes in soil fungal communities may possibly benefit native plants in ecological restoration projects or adversely underlie an exotic shrub's invasiveness.

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Acknowledgments

This research was funded by the Bio-Protection Research Centre (BPRC) and the National Science Challenge, with additional support from the Ross Beever Memorial Mycological Award. I thank the landowners and the Department of Conservation (DOC) for access to the study sites, Xiao Xiao Lin and the team at Massey Genome Service for their support with sequencing, and Ngaire Foster along with the team at Manaaki Whenua for their help with plant nutrient analysis. The research plots at Molesworth Station were initially established by Manaaki Whenua researchers, including D. Peltzer, M. St John, C. Morse and K. Orwin.

I am greatly indebted to my supervisory team, who allowed me to embark on this academic adventure in this beautiful part of the world: Ian, Eirian and Hayley - thank you for all your guidance and support during these last three years and for enabling me to learn many rare lessons. I wouldn't wish for any other group of supervisors. I am likewise grateful that I could both know and work alongside Warwick Allen and all members of the Ecosystem Mycology Group. My thanks goes to all my collaborators and colleagues at the BPRC, to Lauren, Andi, Sam, Rowan, Jacopo, Andrei, Romy, Tom, Isabelle, Francesco, Aimee, Kuchar, Phil and Will.

I am thankful for Angela Wakelin's, Brigitta Kurenbach's and Steven Gieseg's help in the wet-lab, Dave Conder's cheerful assistance within the greenhouses and for the purchasing powers of both Brian Kwan and Angela Langrish. Alan Woods, Linda Morris, Jennifer Bufford and Andrew Holyoake all helped address unexpected pickles and my fieldwork and sample processing wouldn't have been possible without the continued aid of many students, especially Georgia, Nils, Laura and Marcus-Rongowhitiao. I am glad to have been able to work alongside Joanna, Vanita, John, Tyler, Sarah, Jonathan and Zach and to have learnt so much from so many. A special shout-out goes to my academic 'grandparents', to Rus, Yves, Lilia, Nancy, Mia and Jerzy.

My final thanks goes to my parents, to Anna, Christina and Robert for their unending support.

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Chapter 1: General introduction

Background: The invasion of Cytisus scoparius in New Zealand

Invasions by shrubs from the family Fabaceae have occurred as a direct consequence of woodland clearance since the Bronze Age (Figueiral and Bettencourt 2004). This process has been accelerated by the world-wide transport, introduction and subsequent invasion by non-native plants (Jelbert *et al.* 2019, Hill *et al.* 2020). Between 1840 and 2000, over 8 million hectares of New Zealand's native woodland have been replaced with grass- and shrubland (Steens *et al.* 2007). Such large-scale disturbances have often favoured the growth of exotic plants (Fogarty and Facelli 1999, Hierro *et al.* 2005, Christensen *et al.* 2019) and have led to New Zealand's Department of Conservation adopting strategic plans to manage invasive Fabaceae (i.e., legumes) such as *Cytisus scoparius* (Owen 1998). *Cytisus scoparius* (Scotch broom) has colonized a diverse range of habitats worldwide (Potter *et al.* 2009) and in New Zealand (Syrett 2000), especially in the montane North Island and eastern South Island areas (Webb *et al.* 1988). In New Zealand, *C. scoparius* decreases the value of grazing pastures (Prévosto *et al.* 2004) and incurs costs upwards of $100 million per year in control measures and lost productivity (Jarvis *et al.* 2006, Saunders *et al.* 2017). Five biological control agents have already been introduced to New Zealand to stunt the expansion of *C. scoparius* (Syrett *et al.* 1999, Syrett *et al.* 2007, Paynter *et al.* 2012). It is probable that common factors known to promote plant invasion have facilitated the spread of *C. scoparius*, such as disturbance (Sokol *et al.* 2017), agricultural expansion (Chytrý *et al.* 2009, Mariotte *et al.* 2018), the use of superphosphate fertilizers on agricultural land (Smith 1992, Carter *et al.* 2019b), as well as the release of *C. scoparius* from natural predators and pathogens from its native range (Mitchell and Power 2003, Callaway *et al.* 2004, Dickie *et al.* 2017a).

The effect of *C. scoparius* invasion on vegetation and soil nutrient status is profound and long-lasting (Shaben and Myers 2010, Carter *et al.* 2019c) . Whereas *C. scoparius* has a life expectancy of 10-12 years in its native range (Waloff and Richards 1977), it has been found to live as long as 20 years in Australia (Rees and Paynter 1997) and it is possible that *C. scoparius* has an increased seed production (Rees and Paynter 1997, Paynter *et al.* 2016) and an increased pollination rate (Bode and Tong 2018) in exotic environments. *Cytisus scoparius* is capable of growing up to 3 metres in height within 2 years (Watt *et al.* 2003b) and the seeds of *C. scoparius* are able to remain dormant for over 3 years (Bossard 1993) allowing persistent seedbanks to form beneath *C. scoparius* populations (Magda *et al.* 2013). These seedbanks, along with the rapid growth of *C. scoparius*, make *C. scoparius* extraordinarily resistant to eradication, even after applying herbicide (Allen *et al.* 1995, Tran *et al.* 2016, Haubensak *et al.* 2020).

Classified as a drought tolerator (Harrington *et al.* 2018, Míguez-Montero *et al.* 2020), *C. scoparius* exhibits dry climate adaptations such as small deciduous leaves and a durable stem that is able to photosynthesize (Peterson and Prasad 1998), even at temperatures as low as 4°C (Nilsen *et al.* 1993). The association of *C. scoparius* with N-fixing *Bradyrhizobium* (Weir *et al.* 2004) has enabled 30 - 50% of the shrub's long-term nitrogen requirements to be met (Vadakattu and Paterson 2006). Due to these traits, *C. scoparius* is able to grow in areas with either high or low rainfall (Fogarty and Facelli 1999) and is known as a successful pioneer of open habitats that are both deficient in water and N (Williams 1981).

Cytisus scoparius generally increases soil N where it invades (Watt *et al.* 2003a, Haubensak and Parker 2004, Caldwell 2006, Grove *et al.* 2015) (however see Bellingham (1998)'s observation that *C. scoparius* led to soil denitrification) and although it has been suggested that the N-fixing properties of *C. scoparius* may benefit native plants by enriching soil N pools (Caldwell 2006), this benefit is often outweighed by *C. scoparius* competing for available resources (Watt *et al.* 2003b). *Cytisus scoparius* invasion has therefore generally been associated with a decline in native species (Shaben and Myers 2010). In the case of New Zealand, *C. scoparius* can often be found on previously forested pastoral and conservation land (Williams 1981, Bascand and Jowett 1982) alongside other invasive legumes, notably *Ulex europaeus* (gorse) (Reid 1973, Lee *et al.* 1986). Aside from protected conservation areas, *C. scoparius* frequently grows in New Zealand's forestry plantations (Richardson *et al.* 1997, Tran *et al.* 2016, Carter *et al.* 2019a) where it is considered detrimental to the country's pine industry (especially for *Pinus radiata*) as *C. scoparius* can stunt juvenile pine growth and in some cases cause seedling mortality (Watt *et al.* 2003b).

Belowground effects of invasive plants

Although 181 environmental weeds have been mapped in New Zealand (Howell and Terry 2016), among which *C. scoparius* has the 5th largest distribution, only a small proportion of introduced species become invasive in their new range ('invasive' sensu Gaston and Blackburn (2008)). Invasive plants are considered to be one of the most serious threats to local biodiversity and ecosystem functioning (Mooney and Hobbs 2000, Schultheis and MacGuigan 2018), as one of the most common consequences of a plant invasion is a reduction in species richness at the local scale (Powell *et al.* 2011, Essl *et al.* 2019). This loss in species richness is disproportionately rapid on island such as New Zealand (Spatz *et al.* 2017). *Cytisus scoparius* has long been the attention of ecological research yet most commonly with an aboveground focus (Sheppard and Hosking 2000, Syrett 2000, Sheppard *et al.* 2002, Bode and Tong 2018, Bode *et al.* 2019). Relatively less is known regarding the belowground consequences which accompany a plant invasion, with most research in this area taking a plant-soil feedback approach (Mangan *et al.* 2010, Crawford *et al.* 2019). Plants grown in soil from a site where *C. scoparius* has been removed might possibly benefit from N

enrichment (Caldwell 2006) or may adversely have their growth stunted by putatively allelopathic compounds secreted by *C. scoparius* (Grove *et al.* 2012, Pardo-Muras *et al.* 2018, Pardo-Muras *et al.* 2020).

Nitrogen fixation of Cytisus scoparius through rhizobial symbiosis

The presence and composition of soil microbial symbionts has a central influence on both soil nutrient composition and plant-plant interactions (Bever *et al.* 2010) and consequently plant diversity and community structure (Vogelsang *et al.* 2006, Manoharan *et al.* 2017, Semchenko *et al.* 2018). The invasiveness of Fabaceae such as *C. scoparius* can be partially linked to their ability to nodulate (Rodríguez-Echeverría *et al.* 2009). Compared to other N-fixing species, *C. scoparius* is only sparsely nodulated (Helgerson *et al.* 1984), yet can nonetheless exhibit high levels of N-fixation (Watt *et al.* 2003b, Pérez-Fernández *et al.* 2017). As New Zealand has been geographically isolated for 80 million years (Radley 1989), it is postulated that coevolution of native legumes and nitrogen-fixing bacterial symbionts occurred in isolation from other major regions of legume evolution (Weir *et al.* 2004). There is a low diversity in legumes native to New Zealand, which are represented by only 34 species from four genera (Carmichaelia, Clianthus, Montigena and Sophora) (Heenan 1998; Heenan *et al.* 2001). Introduced legumes are likely to outcompete those native to New Zealand.

Despite the shrub's prevalence, *C. scoparius* in New Zealand is more likely to suffer from a scarcity in compatible rhizobia partners as the rhizobial symbiosis is more plant-specific than mycorrhizal symbiosis (Rodríguez-Echeverría *et al.* 2009) (however see Keet *et al.* (2017) regarding how rhizobial symbiosis is not essential for plant invasiveness). Although a scarcity in compatible rhizobia partners has been linked to worldwide establishment or dispersal barriers across multiple legume species (Simonsen *et al.* 2017), legumes which can associate with a broad range of rhizobia are more likely to overcome these barriers (e.g., Klock *et al.* 2015). In New Zealand, all rhizobia found in introduced legumes are from the genus *Bradyrhizobium*, whereas most native legumes associate with the genus *Mesorhizobium* (Weir *et al.* 2004). It is likely that *Bradyrhizobium* was introduced to New Zealand alongside exotic legumes (Warrington *et al*. 2019).

Fungal diversity in New Zealand

Although the study and preservation of fungal biodiversity has garnered less attention compared to other eukaryotes, fungi encompass a substantial amount of global diversity, with fungal diversity estimates ranging from 1.5 to over 165 million species (Hawksworth 2012, Taylor *et al.* 2014, Tedersoo *et al.* 2014, Larsen *et al.* 2017). Censuses of soil fungi have revealed that they are highly diverse, undergo fine-scale niche partitioning (Taylor *et al.* 2014), and that a large proportion is saprotrophic (i.e., decomposers) (Nguyen *et al.* 2016). Rare fungi, particularly ectomycorrhizal

fungi, are sensitive to the processes of disturbance (Dickie and Reich 2005, Dickie *et al.* 2009) and N deposition (Avis *et al.* 2008), which both accompany *C. scoparius* invasion in New Zealand.

Fungal diversity is of integral as well as economic and cultural importance. Rare fungal species may be important sources of future pharmaceuticals (Chen *et al.* 2019b). In New Zealand, fungi are used by Māori as material to create traditional tattoos (Fuller *et al.* 2004, Dickie *et al.* 2020), have the potential to be used as biocontrol agents (Kuchár *et al.* 2019, Ehlers *et al.* 2020) and are deemed important to the country's wine (Knight *et al.* 2020) and food industry (Guerin-Laguette *et al.* 2020).

Belowground effects of invasive plants: Arbuscular mycorrhizal fungi

Among the major taxa of soil fungi, the sub-phylum Glomeromycotina has a world-wide distribution (Öpik *et al.* 2006) and is an important component of belowground microbial communities, with ~80% of land plants being dependent on Glomeromycotina (Davison *et al.* 2015), including most invasive woody plants (Rejmánek and Richardson 2013). Along with some members of the sub-phylum Mucoromycotina (Orchard *et al.* 2017, Walker *et al.* 2018), Glomeromycotina are also known as arbuscular mycorrhizal fungi (AMF). Despite AMF's global prevalence, there are only ~250 morphologically defined and 350 to 1000 molecularly defined AMF species (Davison *et al.* 2015). Dependent on C from plant roots, AMF confer different benefits to various plant species (Klironomos 2003, Lefebvre 2019), mainly by facilitating nutrient uptake, particularly P, although AMF are functionally quite diverse (Munkvold *et al.* 2004, Rivero *et al.* 2018) and may increase a plant's tolerance to drought and root pathogens. Granted that most AMF are in fact mutualists (Pringle *et al.* 2009), there is substantial variation as to what extent AMF benefit hosts, and some plant-AMF associations have been known to reduce plant fitness (Jones and Smith 2004, Hoeksema *et al.* 2018, Dierks *et al.* 2019). It has become apparent that AMF communities are not random assemblages (Davison *et al.* 2011). Distinct communities of AMF are frequently formed around a given plant species (Van den Koornhuyse *et al.* 2003) which can impact the composition of aboveground plant communities (Tedersoo *et al.* 2020) in a "bottom-up" manner (Hartnett and Wilson 1999, Van der Heijden and Horton 2009).

Due to the relatively low host-specificity of AMF (Nuñez and Dickie 2014), many arbuscular mycorrhizal plants may not require AMF from their native range, but can integrate into existing ecological networks by forming novel associations with native AMF (Brundrett 2009, Zhang *et al.* 2017). AMF association can be an important pathway through which invasive plants alter the performance of native species (Hawkes *et al.* 2006, Menzel *et al.* 2017). It is known that a host plant changing its species of AMF has the potential to impact decomposition rates (Hodge *et al.* 2001, Gui *et al.* 2017) and plant nutrient uptake (Cavagnaro *et al.* 2005, Ingraffia *et al.* 2019) among other effects. Therefore, should an invasive plant alter an AMF community, this could potentially lead

to broader changes in general soil microbial community composition and structure, as well as affect resource availability and nutrient cycling (Asner *et al.* 2008, Rascher *et al.* 2011).

Belowground effects of invasive plants: Soil pathogens

The benefits of AMF can be outweighed by an opposing process such as the presence of soil pathogens. As an example, it was found that the application of fungicide to natural populations of *Vulpia ciliata* had no effect on plant performance (Newsham *et al.* 1995), which was due to a simultaneous reduction of AMF and pathogenic fungi in roots. Belowground pathogens can greatly reduce reproduction, growth and survival of plants (Burdon 1987, Raaijmakers and Mazzola 2016) and many pathogens may be host-specific (Van der Putten *et al.* 2007a, Bakker *et al.* 2018). Despite soil pathogens playing a major role in structuring plant community compositions (Latz *et al.* 2016), their inclusion in ecosystem studies has often been overlooked (Beckerman and Petchey 2009), which can lead to an ecosystem's complexity being underestimated (Wood *et al.* 2007). Soil pathogens not only affect host plants, but also interact with other soil microorganisms (Spagnoletti *et al.* 2017).

One main reason why invasive plants can benefit from being introduced in a new environment is that the plants may encounter fewer host-specific pathogens compared to their native range (Reinhart and Callaway 2006, van der Putten *et al.* 2016). For example, it was found that the grass *Ammophila arenaria*, which is native to Europe, encountered fewer nematode species in New Zealand relative to its native range (Van der Putten *et al.* 2005). This may not always be the case, as the presence of strong indigenous belowground pathogens may also form a barrier to invasion (Reinhart and Callaway 2006). Such strong indigenous pathogens could account for a large number of seldom studied failed plant invasions and unsuccessful attempts to grow agricultural crops (Zenni and Nuñez 2013). There is limited information on soil pathogens closely associated with *C. scoparius*, although *Cytisus* sp. in North America have been observed with known soil pathogens such as *Pythium* sp. and *Rhizoctonia* sp. (Farr *et al.* 1989).

Use of eDNA metabarcoding in community analysis

Detecting often cryptic belowground mutualists has traditionally been difficult (Atkins and Clark 2004) reliant on specialist taxonomic knowledge of belowground biota (McCartney *et al.* 2003). In contrast, using molecular-based detection methods to identify soil biota yields more reproducible and rapid results (Aslam *et al.* 2017, Nilsson *et al.* 2019a) (although see Malarczyk *et al.* (2019) regarding alternative methods). An increasingly popular molecular method is metabarcoding, which combines DNA-based identification with high-throughput sequencing, allowing numerous species within an environmental sample to be assessed (Taberlet *et al.* 2012). To address the increase in metabarcoding-based studies of environmental DNA (eDNA), several methodological

reviews have been published on the molecular and bioinformatical steps involved (e.g., Hiraoka *et al.* (2016), Lear *et al.* (2018)), yet comparatively less attention has been given to the systematic and reproducible processing of eDNA samples prior to sequencing (Dickie *et al.* 2018).

Thesis aim and research objectives

The goal of my thesis was to study the effects of soil under various levels of *C. scoparius* invasion and then examine whether the unique soil legacy of *C. scoparius* was contingent on how *C. scoparius* shaped fungal communities in soil. Knowing the putative cause of the soil legacy of *C. scoparius*, I then analysed whether a plant's direct and indirect responses to *C. scoparius* are observable outside of the greenhouse environment under field conditions.

I began my research by studying the effect of the soil legacy of *C. scoparius* in a controlled environment (via a greenhouse experiment). Knowing the effect of *C. scoparius'* soil legacy under regulated conditions, I then undertook a field survey, in which I systematically recorded changes in fungal community composition across a natural density gradient of *C. scoparius* invasion. I subsequently studied how different fungal communities found near *C. scoparius* may underlie the results of my greenhouse experiment. Knowing how changes in the composition of soil fungi play a significant role in the soil legacy of *C. scoparius*, I measured the importance of the belowground associations of *C. scoparius* by examining the growth of plants in the presence of live *C. scoparius* (via a field-based experiment). As a final step, I investigated whether the eDNA metabarcoding techniques I used could be optimised for future researchers (via a methodological experiment).

The objectives of my thesis are encompassed in five sections:

- Via a **greenhouse experiment**, to study the soil legacy of *C. scoparius* on plants with varying species traits to determine whether *C. scoparius* invasion facilitates the spread of other introduced plants and/or species from the taxonomic family of *C. scoparius* (Chapter 2).
- Conduct a **field survey** to obtain insight into specific changes to fungal communities induced by *C. scoparius* belowground, which may contribute to the invasion success of *C. scoparius* (Chapter 3).
- Perform a **methodological experiment** examining the benefits and downsides of pooling metabarcoded fungal eDNA samples (Chapter 4).
- Based on the results of Chapters 2 & 3, examine via mixed-effect **modelling** whether the influence of the soil legacy of *C. scoparius* on other plants may be attributed to specific fungal communities (Chapter 5).
- Undertake a **field-based experiment** to quantify the relative importance of the direct and indirect components of the belowground impact of *C. scoparius* on plant growth (Allen *et al.* (2020); Appendix E).

Chapter 2: The soil legacy of *Cytisus scoparius*

Abstract

Plant invasion can cause changes in soil community composition and soil nutrient availability which may drive the composition of subsequent plant communities, particularly when the invasive plant is able to fix nitrogen. The effect of plant invasion can persist after removing the introduced plant and may be specific to individual species, which in turn may affect the success of ecological restoration projects. Using soil extracted from across a density gradient of exotic *Cytisus scoparius* (Fabaceae), I tested whether the shrub's soil legacy favoured the growth (dry biomass) and nutrient acquisition (shoot % N and shoot % P) of a selection of plants native and exotic to New Zealand, which were either able or unable to fix nitrogen. I found that, compared with uninvaded soil, plants grown in *C. scoparius*-invaded soil had 1) higher above- and belowground biomass and higher total N:P ratios, particularly for native plants, 2) lower root:shoot ratios, 3) no apparent changes in shoot % P, and 4) a mixed response concerning shoot % N, where *C. scoparius* coverage did not change shoot % N in any tested Fabaceae species, yet affected half of the tested non-Fabaceae. The soil legacy of *C. scoparius* in a controlled greenhouse environment favoured the growth of non-leguminous native New Zealand plants over its own taxonomic family, despite prior field studies suggesting the opposite. Having found that the predominantly positive soil legacy effect of *C. scoparius* could only be partly attributed to soil chemical traits, microbial effects likely play an important role in the invasion success of *C. scoparius.*

Keywords

Cytisus scoparius, facilitation, functional traits, invasive species, removal effects, seedling establishment

Introduction

During early development, plant seedlings interact primarily with a soil environment moulded by past generations of plants. These older established plants would have interacted with and shaped communities of soil microorganisms which in turn interact with the newly recruited seedlings (Bever *et al.* 2010, Kulmatiski and Beard 2011). Soil microorganisms, such as fungi or bacteria, often have significant positive and negative effects on plants through root-rhizosphere mutualism (Brundrett 1991), pathogen effects (Packer and Clay 2000, Latz *et al.* 2016) and by driving nutrient cycles (Horwath 2017).

Plant-soil feedback, the process whereby a plant's effect on soil community and the environment influences the growth of future generations of plants, is generally considered to be an important contributing cause of plant rarity and invasiveness in communities (Klironomos 2002). For a given plant species, soil obtained from closely related plants generally has a more negative effect on plant growth than soil obtained from distantly related plants (Kempel *et al.* 2018). A plant's invasive status often correlates with more positive (or less negative) levels of plant-soil feedback (Kulmatiski *et al.* 2008), although both positive and negative plant-soil feedbacks have been reported for invasive plants (Reinhart *et al.* 2003, Suding *et al.* 2013, Dostálek *et al.* 2016, Aldorfová *et al.* 2020).

Positive plant-soil feedbacks can occur when certain plants accumulate specific microorganisms near their roots that benefit the plant, such as N-fixing bacteria or arbuscular mycorrhizal fungi. It has been observed that positive feedbacks lead to a loss of local community diversity (Bever *et al.* 1997, Bever 2002), although it has likewise been proposed that positive feedbacks are unlikely to cause a loss of diversity (Dickie *et al.* 2014). One form of negative plant-soil feedback occurs when pathogens accumulate in the rhizosphere of plant species. Pathogen accumulation in non-native plant species usually increases with residence time (Diez *et al.* 2010, van Kleunen *et al.* 2018). Negative plant-soil feedbacks can enhance plant community diversity by increasing species turnover rates (Klironomos 2002, Teste *et al.* 2017). According to a meta-analytical review by Kulmatiski *et al.* (2008), plant-soil feedbacks most commonly have medium to large negative effects on plant growth (with the scale of negative effects outweighing that of positive effects).

Cytisus scoparius (Scotch broom) is a leguminous shrub difficult to control with herbicides (Tran *et al.* 2016, Haubensak *et al.* 2020) which has colonized a diverse range of habitats worldwide (Holm *et al.* 1997, Brandes *et al.* 2019) and in New Zealand (Parsons and Cuthbertson 1992, Tran *et al.* 2016). In New Zealand, *C. scoparius* can often be found in low-intensity grazing grasslands alongside other invasive legumes, notably gorse (*Ulex europaeus*) (Lee *et al.* 1986, Ghanizadeh and Harrington 2019). Large scale invasions by species from the family Fabaceae have occurred as a direct consequence of woodland clearance since the dawn of agriculture (Figueiral and Bettencourt

2004). Such an invasion is happening in New Zealand at substantial economic costs (Saunders *et al.* 2017) and it is probable that common factors known to promote plant invasion have facilitated the spread of *C. scoparius*, such as disturbance (Sokol *et al.* 2017, Daniels and Larson 2020), agricultural expansion (Mariotte *et al.* 2018), the use of superphosphate fertilizers on agricultural land (Carter *et al.* 2019b) as well as the release of *C. scoparius* from fungal and viral pathogens from its native range (Mitchell and Power 2003, Dickie *et al.* 2017a). *Cytisus scoparius* invasion is associated with an increase in exotic species and/or a decline in native species, which correlates with the ability of *C. scoparius* to modify soil nutrient availability (Shaben and Myers 2010, Carter *et al.* 2019c). Given the long-lasting effects that *C. scoparius* invasion may have on surrounding soil, the shrub's belowground impact on New Zealand plants deserves more attention.

Nitrogen (N) and phosphorus (P) are the two most limiting nutrients in terrestrial ecosystems (Han *et al.* 2005, Vitousek *et al.* 2010). Although minor soil denitrification caused by *C. scoparius* has been documented (Carter *et al.* 2019c), the species is commonly known as an N-fixer and can fix as much as 111 kg N ha^{-1} per year into aboveground tissues (Watt *et al.* 2003b). Once *C. scoparius* is removed from a site, soil affected by *C. scoparius* might act as a nursery plant for other species, specifically those which are unable to fix their own N. In addition to fixing N, *C. scoparius* increases soil organic C (Fogarty and Facelli 1999) and soils under *C. scoparius* have been found to have higher activities of two soil enzymes which are involved in P-acquisition (Caldwell 2006). Increases in the availability of soil P have been associated with *C. scoparius* invasion (Dewar *et al.* 2006), yet decreases in available P have likewise been recorded (Shaben and Myers 2010, Slesak *et al.* 2016).

The effect of the soil moulded by *C. scoparius* on other plants has been shown to be species-specific. On one hand, the N-fixing properties of *C. scoparius* might have enabled the spread of other invasive species such as hawthorn (*Crataegus monogyna*) (Williams *et al.* 2010) and *C. scoparius* presence has been associated with an increase in the cover of exotic sweet vernal grass (*Anthoxanthum odoratum*) (Carter *et al.* 2019c). On the other hand, *C. scoparius*, whether live or removed, is known to decrease the abundance of ectomycorrhizal fungi leading to an overall negative effect on seedling growth of certain pine (*Pseudotsuga menziesii)* (Grove *et al.* 2012). Assuming longer-term persistence of the effect of *C. scoparius* within this chapter, I refer to soil conditioned by *C. scoparius* as being soil representative of the soil legacy of *C. scoparius*.

The design of soil legacy studies requires careful handling. Studies with natural gradient approaches (i.e., which test various levels of plant invasion) are needed to better forecast how short-term plant-soil feedbacks can expand from species-level to long-term ecosystem dynamics (Kardol *et al.* 2012, Kardol *et al.* 2013). Although plant-soil feedbacks have been assumed to affect plant growth in a linear manner (Bever *et al.* 1997, Bever 2003), non-linear plant-soil feedback

effects can be overlooked if linearity is assumed when designing an experiment (Hawkes *et al.* 2013). All published studies on the effects of *C. scoparius'* soil legacy revolve around a small number of plants and soils and none look at how different degrees of *C. scoparius* invasion affect plant growth.

Among the studies on the soil legacy *of C. scoparius*, Haubensak and Parker (2004) found that *C. scoparius* "may have inhibitory effects on some plants growing in invaded soils", yet their study was restricted to a single plant (*Achillea millefolium*). A further study by Grove *et al.* (2015), which reported negative *C. scoparius* soil legacy, was also restricted to *Pseudotsuga menziesii.* Other studies have examined *C. scoparius'* soil legacy, yet not in context with plant growth. Davis (2018) studied *C. scoparius* soil legacy using two different soils and only one plant (*C. scoparius*) and found that *C. scoparius* seedlings grew smaller in soil with *C. scoparius* legacy.

In a field-based experiment (Allen *et al.* (2020); Appendix E), we found that live *C. scoparius* facilitated the growth of both native and exotic legumes, while slightly favouring exotic legumes. Here I aimed to examine how both native and exotic plants respond to *C. scoparius'* soil legacy and whether changes in soil composition induced by an invasive N-fixing plant might potentially promote the growth of co-invading plants from its own taxonomic family (Fabaceae) post removal. I also aimed to examine whether changes in soil composition induced by an invasive N-fixing plant might potentially promote the growth of non-leguminous plants, a functional group which was not included in our field experiment (Allen *et al.* (2020); Appendix E). Here I specifically use an approach which observes the growth of plants grown in soil with differing degrees of *C. scoparius* soil legacy, rather than a more common presence/absence approach where a plant is grown in either a 'Control' or 'Effect' soil.

I hypothesized that:

- *Cytisus scoparius* has soil legacies influencing the growth of other plants which differ according to plant species.
- The soil legacy of *C. scoparius* will have a more positive effect on exotic plants than on natives.
- As *C. scoparius* frequently co-occurs with other invasive members of the Fabaceae (notably *Ulex europaeus*), that *C. scoparius'* soil legacy will have a more positive effect on members of its own taxonomic family when compared to unrelated plants.

To test these hypotheses, I use dry biomass (root and shoot) and nutrient analysis data (shoot % N and % P) from plants of varying functional groups (half native to New Zealand and half from the family Fabaceae) which were grown in soils collected from across a *C. scoparius* density gradient.

With accompanying data on soil chemical composition, I will have a better ability to tease apart the chemical and biological properties underlying *C. scoparius'* soil legacy.

Methods

Study site and soil collection

I carried out the experiment in a single glasshouse at the University of Canterbury (Christchurch, New Zealand). I collected soil from 18 permanent sampling plots in the Saint James Conservation Area at Molesworth Station, located in the Hurunui district of the South Island of New Zealand. Each permanent sampling plot was modelled on field protocols outlined by Hurst and Allen (1993) and was previously laid out by Manaaki Whenua. The same plots have been used in previous experiments (Broadbent *et al.* 2017) and data on soil chemistry is available for all plots. *Cytisus scoparius* is widely spread throughout this region and the chosen plots range from areas uninvaded by *C. scoparius* to near mono-dominant *C. scoparius* patches. All plots were situated within 2.5 km of each other at an altitude 872-933 m above sea level. A map pinpointing the plots is shown in Figure 1 (plot coordinates are listed in Appendix A1). I collected data on *C. scoparius* density for each plot, including *C. scoparius* coverage estimates and the distance from the extracted soil to the closest mature *C. scoparius*.

During a 10 day period in November 2017, I extracted 25 L of the uppermost 150-200 mm of soil close to the centre of each of the 18 plots using spades sterilized in a 10% v/v bleach solution (8% sodium hypochlorite in undiluted bleach) for >10 minutes (Prince and Andrus 1992). The exact location of the collected soil from each plot is shown in Figure 2. The soils were kept in separate clean plastic bags according to plot. Subsequent soil processing at the University of Canterbury was performed within 2 weeks to minimize changes in soil microbial composition which occurs during prolonged soil storage, thus encouraging a 'living' effect of the soil on plants. Each soil was broken up with bleach-sterilized spading forks and all stones above 20 mm in diameter were manually removed as well as any large woody roots and plants. I then mixed the loosened soil at a 1:1 volume ratio with washed river sand. This mixing was done to minimize the quantity of soil required (and thereby any disruption caused to the permanent sampling plots) as well as to help standardize soil porosity. The soil-sand mixtures were used to fill bleach-sterilized ~1.4 litre pots with drainage holes (110 mm length × 110 mm width × 120 mm height), each containing 200-250 g of stone chips (10 mm - 20 mm) at the bottom of the pots to improve drainage.

Plant selection and glasshouse experimental layout

Sixteen plant species were used in this experiment (Table 1), each planted into each of the 18 collected soils giving a total of 288 samples. Half the plant species were native to New Zealand, the other half introduced to New Zealand. Half of the plant species are members of the Fabaceae, whereas the other half were chosen from four taxonomic families (Myrtaceae, Pinaceae, Poaceae

and Podocarpaceae). The selected plants can associate with arbuscular mycorrhizal fungi and ectomycorrhizal fungi, and in some cases with both (e.g., *Leptospermum scoparium*) (McKenzie *et al.* 2006) or neither (*Lupinus arboreus*) (Oba *et al.* 2001). Prior to being planted in their respective soils, some of the plants underwent stratification and/or scarification (according to the seed supplier's recommendations) to improve germination rates. Except for the exotic grasses *Agrostis capillaris* and *Anthoxanthum odoratum,* all plants were germinated in propagation trays filled with a 1:1 mixture of vermiculite and perlite under greenhouse conditions.

During transfer of seedlings into the collected soils, an additional step was taken to account for differences in unique biota which could potentially be present in different propagation trays. Two live plant specimens and ~20g vermiculite-perlite mixture were taken from each propagation tray and mixed with tap water. The resulting slurry was then added in equal amounts to each pot, so that each sample was inoculated with the same propagation tray biota at the beginning of the growth period.

Due to the fast-growing nature of *A. capillaris* and *A. odoratum*, seeds of these grasses were directly sown on the prepared soils. For *A. capillaris*, it was difficult to reliably separate between individual plants, hence all plants that were not within a 30 mm diameter circle in the pot's centre were weeded out. For *A. odoratum*, most pots yielded three to five distinct grasses, all of which were weeded out of the pot within the first 2 weeks except for one randomly chosen individual. Two *Trifolium repens* seedlings were initially planted in each pot. This additional step was done due to the delicate consistency of the seedlings, which could be damaged when transplanted from the vermiculite-perlite propagation trays into the soil pots. In the case where both *T. repens* seedlings survived the first two weeks in soil, one randomly chosen shoot was pulled out with its roots.

I laid out all pots on a single bench within a single glasshouse in a completely random design with ~100 mm between pots (Figure 3). Each pot stood on a ~20 mm tall upturned pot saucer which both aided water drainage and insured that water passing through the pots could not contaminate adjacent pots. The pots remained in the same position throughout the course of the experiment and were watered regularly using an overhead mist-propagation system. Anti-drip nozzles were used to prevent falling water drops splashing wet soil from one pot into another, potentially causing an unwanted cross-contamination of soil biota or minerals. Although early plant mortality proved low, any obviously dead plants were replaced within the first 6 weeks of the experiment. Plants that died after six weeks were not replaced. All pots were weeded every week throughout the growing period, which lasted ~7 months from early December 2017 to harvest in the beginning of June 2018.

The height of the plants was measured in early December immediately after planting, then in early March and lastly in late May prior to harvest. The December height measurement for *A. capillaris*

and *A. odoratum* was taken as zero, as seeds for these plants were directly sown onto soil and had just started germinating. Throughout the course of the experiment, no obvious plant herbivory was noticed during regular weeding, although a few plants showed minor chlorosis.

Plant harvesting and nutrient analysis

Live plants (n = 277) were destructively harvested in order to obtain above- and belowground dry biomass in the beginning of June 2018. Any deceased plants (n = 11) were excluded from further analysis. Harvesting was undertaken by washing plant roots in a basin of running water and drying the above- and belowground plant biomass in separate paper bags at 55°C for a minimum of 72 hours in aerated laboratory drying ovens prior to weighing. For *A. capillaris*, which was difficult to grow individually, the above- and belowground biomass of the tallest individual was measured. The exposure time of dried plants was kept to a maximum of 15 minutes when taking them out of the drying ovens for weighing, thus minimizing changes in weight caused by hydration.

Roots obtained from certain plant species (*A. odoratum* and *A. capillaris* in particular) and certain soils (e.g., plot MW17) proved difficult to wash appropriately and still contained some soil material entangled in their roots when their dry biomass was measured. For each plant, the presence of other material entwined in the roots was noted as 'None' (91.7%), 'Minor' (3.6%) or 'Major' (4.7%).

Of the 277 harvested plants, all shoots weighing >0.05 g (n = 262) were sent for P and N quantification via the Kjeldahl method (Bradstreet 1954) at Manaaki Whenua's Environmental Chemistry Laboratory in Palmerston North, New Zealand. Shoots weighing <0.05 g (9% of total samples, n = 26) did not have the minimum amount of biomass required for accurate nutrient analysis and were therefore discarded. Shoots weighing >0.5g (40.6% of total samples, n = 117) were mechanically ground into a fine powder at the University of Canterbury. I pulverised all available aboveground biomass (i.e., the whole shoot) using a Retsch® MM301 mixer mill fitted with cleaned 50 mL grinding jars for 30 seconds at 30Hz. Shoots which were too large to be crushed in a single jar were ground throughout the course of several sessions, then combined. Shoots weighing 0.05-0.5 g were small enough to be ground by hand at Manaaki Whenua prior to nutrient analysis.

Statistical analysis

I used R version 3.5.0 (Team 2013) for creating graphs and conducting analyses alongside the R package "dyplr" (Wickham *et al.* 2015). I excluded plants found dead at harvest (3.8% of total) and when calculating root and total biomass, I only used samples with completely clean roots (91.7% of live plants). I chose to use clean roots only, as the correlation between root mass and shoot mass of all harvested live plants ($R^2 = 0.0108$; $P < 0.0001$) increased markedly after removing dirty roots

($R^2 = 0.2747$; $P < 0.0001$). To quantify the effects of soil under various levels of *C. scoparius* invasion on plant biomass and nutrient composition according to plant species traits, I used linear mixed-effect models via the R package "lme4 (v1.121)" (Bates *et al.* 2014), setting plant origin, legume status and ectomycorrhizal status as fixed effects and sampling plot and plant species as random effects. The mixed models explicitly separate variation between-plant species and within-plant species, thus enabling *C. scoparius'* effect on naturally larger plants to be compared with naturally smaller plants (Millar and Anderson 2004). I log-transformed the measurements I collected for "distance from extracted soil to closest *C. scoparius*".

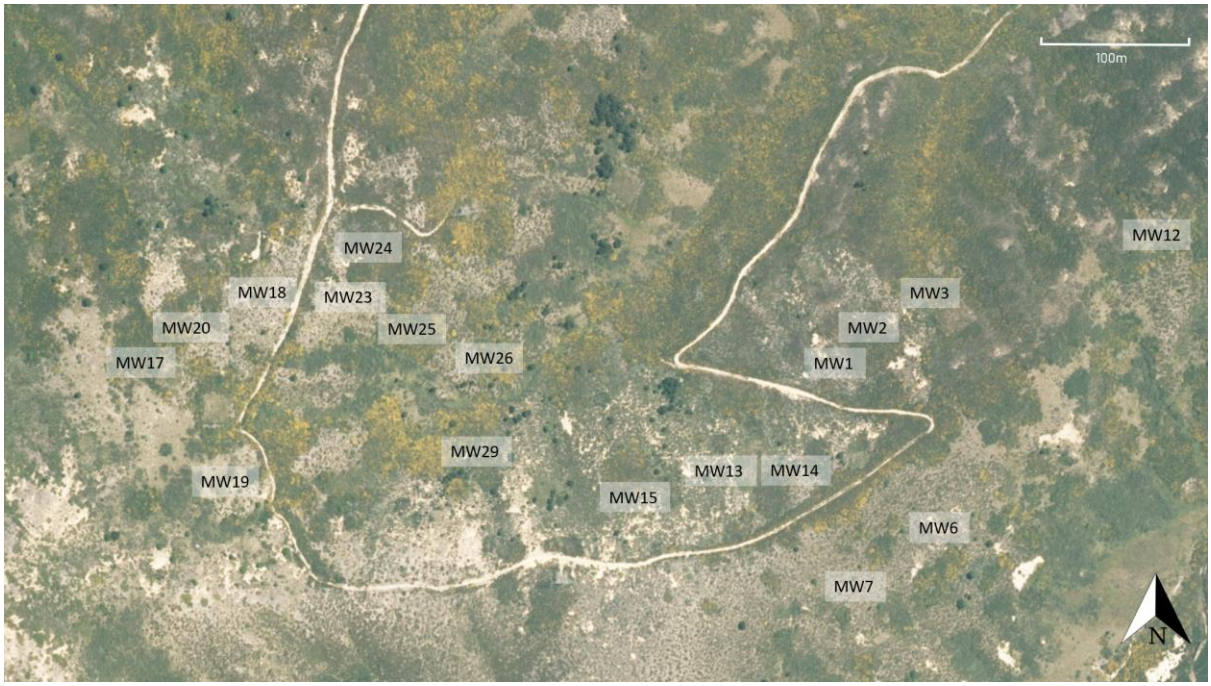


Figure 1. ArcGIS image of 18 plots in Molesworth used for soil collection (© ArcGIS, Environmental Systems Research Institute). The yellow areas in the image are predominantly populated by *C. scoparius*.

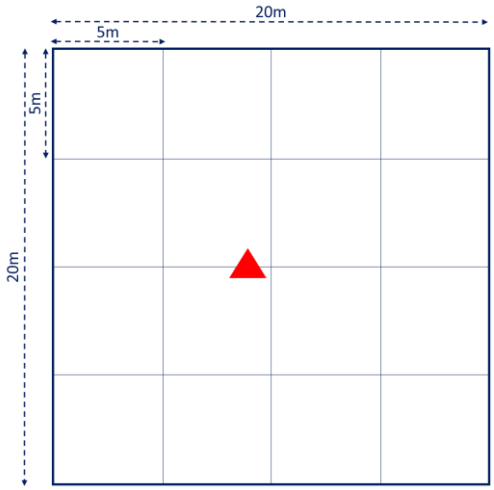


Figure 2. Position of soil sample extracted from each sampling plot (red triangle). *Cytisus scoparius* coverage estimates were measured throughout the 20 × 20 m plot as well as the distance from the extracted soil sample to the closest *C. scoparius*.

Table 1. List of plant species used and their status as native or introduced to New Zealand. AMF = Arbuscular mycorrhizal fungi. ECM = Ectomycorrhizal fungi. Seeds were collected in person or bought from NZ Seeds (www.nzseeds.co.nz) or Topseeds (www.topseedsltd.com). *Lupinus arboreus* has been considered as non-AMF (Oba *et al.* 2001). *Leptospermum scoparium* has been known to form both AMF and ECM associations (McKenzie *et al.* 2006). Although *Pinus radiata* and *Pseudotsuga menziesii* are primarily ectomycorrhizal, both can form AMF associations (Teste *et al.* 2020).

	Scientific Name	Common Name	Fabaceae	Plant Class	AMF
Introduced to N.Z.	*Cytisus scoparius*	Scotch broom	✓	Legume	✓
	Trifolium repens	White clover	✓	Legume	✓
	Ulex europaeus	Gorse	✓	Legume	✓
	Lupinus arboreus	Yellow bush lupine	✓	Legume	✘
	Agrostis capillaris	Browntop	✘	Grass	✓
	Anthoxanthum odoratum	Sweet vernal grass	✘	Grass	✓
	Pinus radiata	Monterey pine	✘	Tree	*Trace* (+ECM)
	Pseudotsuga menziesii	Douglas Fir	✘	Tree	*Trace* (+ECM)
Native to N.Z.	*Sophora microphylla*	Kōwhai	✓	Legume	✓
	Clianthus puniceus	Kaka beak	✓	Legume	✓
	Sophora tetraptera	Large-leaved kōwhai	✓	Legume	✓
	Carmichaelia australis	N.Z. broom	✓	Legume	✓
	Chionochloa conspicua	Tussock grass	✘	Grass	✓
	Poa colensoi	Tussock	✘	Grass	✓
	Podocarpus totara	Tōtara	✘	Tree	✓
	Leptospermum scoparium	Mānuka	✘	Shrub	✓ (+ECM)

Figure 3. Set-up of the experiment at a University of Canterbury glasshouse. Each individual pot was elevated above the table to avoid cross-contamination of soil biota after mist-watering.

Results

In total, 277 live plants were harvested. When grouped according to plant origin and species functional group, exotic Fabaceae had both the largest shoot mass and tallest height (Table 2).

Effect of C. scoparius coverage on biomass

There was a significant three-way interaction for shoot biomass between *C. scoparius* coverage, plant origin, and legume status as well as *C. scoparius* coverage, plant origin and ectomycorrhizal status (Table 3; accompanying *t*-values in Appendix A2). With the exception of *Sophora microphylla*, the other seven plant species native to New Zealand showed greater aboveground dry biomass when grown in soil with *C. scoparius* legacy (Figure 4). The soil legacy of *C. scoparius* only favored half of the introduced plant species. All non-N-fixing native New Zealand plants in the experiment (n = 4) showed increased biomass under the influence of *C. scoparius'* soil legacy, compared with only 1 out of 4 exotic non-N-fixing species. In general, plants from the family Fabaceae only slightly favoured growing in soil with *C. scoparius* legacy, with 6 out of 8 species showing increased aboveground dry biomass compared with 5 out of 8 plant species which do not fix N.

When substituting *C. scoparius* coverage with the log-transformed distance to the closest mature *C. scoparius*, there were some minor qualitative differences in results (Appendix A3), yet all 3-way interactions remained significant. Similar species trait-specific increases in biomass over *C. scoparius* coverage could also be observed for dry root biomass, total dry biomass and plant height at harvest (Appendix A4) and there was little qualitative difference in results when *C. scoparius* % coverage was substituted with distance from soil extracted to closest mature *C. scoparius* (Appendix A5). The inclusion of either deceased or replaced plants had a negligeable effect on results. The effect of *C. scoparius'* soil legacy on shoot biomass was generally most pronounced when soil was extracted within ~2 metres of mature *C. scoparius*.

There was a significant one-way interaction for log-transformed root:shoot (g) ratio and *C. scoparius* coverage (t = -3.094; P = 0.0019). As *C. scoparius* coverage increased, root:shoot (g) ratio decreased for 2 native plants (i.e., *Podocarpus totara*, R^2 = 0.2117; P = 0.0481; and *Sophora tetraptera*, R^2 = 0.2765; P = 0.0146).

Effect of C. scoparius coverage on shoot % N and shoot % P

No significant effect was observed when analysing shoot % P over *C. scoparius* coverage. There was a significant three-way interaction for shoot % N between *C. scoparius* coverage, plant origin, and legume status (t = 2.464; P = 0.0137; Table 3). Results for shoot % N over *C. scoparius* coverage

varied. There were no significant effects for any Fabaceae, yet half of the tested non-Fabaceae reacted both positively and negatively to *C. scoparius*. *Cytisus scoparius* coverage increased shoot % N of *Agrostis capillaris*, *Pinus radiata* and *Podocarpus totara*, yet decreased shoot % N of *Anthoxanthum odoratum* (Figure 5).

There was a significant two-way interaction for shoot N:P (%) ratio between *C. scoparius* coverage and plant origin (t = -3.320; P = 0.0009; Table 3). Shoot N:P (%) ratio over *C. scoparius* coverage decreased for 2 out of 8 native plants and increased for a single exotic plant (Figure 6). Shoot N:P (%) ratios for exotic Fabaceae were notably above other plants.

Effect of C. scoparius coverage on total shoot N:P

There was a significant three-way interaction for total shoot N:P ratio between *C. scoparius* coverage, plant origin and legume status (t = -4.907; $P < 0.0001$; Table 3) as well as between *C. scoparius* coverage, plant origin and ectomycorrhizal status (t = 2.848; P = 0.0044; Table 3). With the exception of *Clianthus puniceus*, all plants which had shown a significant increase in aboveground biomass as *C. scoparius* coverage increased (i.e., Figure 4) likewise showed a significant increase in total shoot N:P ratio over *C. scoparius* coverage (Figure 7).

Effect of soil chemistry

Cytisus scoparius coverage had no detectable effect on soil chemistry (Appendix A6). In some instances, there was an effect of soil Ca (cmol(+)/kg) and soil Mg (cmol(+)/kg) on plant morphology, yet for all tested plant response variables, the effect of *C. scoparius* coverage superceded that of soil chemistry, both in terms of t-value and significance (Table 4). No effect was found for shoot % N over soil N(%). Uniquely for *Ulex europaeus*, there was a significant positive effect (post Bonferroni correction) of soil Olsen P (mg/kg) on shoot % P (R^2 = 0.6801; $P < 0.0001$). Also uniquely for *Ulex europaeus*, there was a significant negative effect (post Bonferroni correction) of soil Olsen P (mg/kg) on shoot N:P (%) ratio (R^2 = 0.6011; $P < 0.0001$) and a significant negative effect of soil K (cmol(+)/kg) on shoot N:P (%) ratio (R^2 = 0.4792; P = 0.0012).

Table 2. Overview of mean plant measurements (± standard errors) for each individual species and all plants (n = 277) grouped according to species functional groups (see Table 1). AMF = Arbuscular mycorrhizal. ECM = Ectomycorrhizal. Across all examined plant species, *C. scoparius* on average had the highest shoot height (535.6 ± 60.27 mm), largest shoot mass (2.240 ± 0.42 g), highest total shoot N (5.22 ± 0.86 gN) and highest total shoot P (0.26 ± 0.04 gP). *Cytisus scoparius'* total shoot N and total shoot P were markedly above the average of all other exotic Fabaceae (total shoot N = 2.83 ± 0.44 gN; total shoot P = 0.17 ± 0.03 gP), which in turn were above all native Fabaceae (total shoot N = 1.15 ± 0.2 gN; total shoot P = 0.10 ± 0.01 gP). Plants which solely formed arbuscular mycorrhyzal associations had a 2.79 times higher mean total N (1.59 ± 0.16 gN) compared with ectomycorrhyzal plants (*Leptospermum scoparium*, *Pinus radiata*, *Pseudotsuga menziesii*; mean total N = 0.57 ± 0.09 gN).

	Number of live plants harvested	Shoot height (mm)	Shoot mass (g)	%N	%P
Agrostis capillaris	18	174.8 ± 11.09	0.093 ± 0.01	1.43 ± 0.10	0.16 ± 0.01
Anthoxanthum odoratum	18	267.7 ± 10.34	0.703 ± 0.12	1.65 ± 0.09	0.22 ± 0.02
Carmichaelia australis	17	420.6 ± 51.06	0.911 ± 0.18	1.53 ± 0.09	0.14 ± 0.01
Chionochloa conspicua	18	293.8 ± 29.67	0.284 ± 0.06	1.71 ± 0.04	0.17 ± 0.01
Clianthus puniceus	16	190.2 ± 38.24	0.538 ± 0.23	2.82 ± 0.13	0.24 ± 0.03
Cytisus scoparius	17	535.6 ± 60.27	2.240 ± 0.42	2.30 ± 0.06	0.12 ± 0.01
Leptospermum scoparium	17	277.5 ± 21.56	1.021 ± 0.18	1.14 ± 0.09	0.13 ± 0.01
Lupinus arboreus	13	143.1 ± 29.08	0.295 ± 0.10	2.17 ± 0.08	0.08 ± 0.01
Pinus radiata	18	121.1 ± 13.32	0.364 ± 0.08	1.21 ± 0.08	0.16 ± 0.02
Poa colensoi	17	278.1 ± 15.72	0.821 ± 0.23	1.33 ± 0.11	0.15 ± 0.01
Podocarpus totara	18	137.8 ± 16.32	0.335 ± 0.05	1.15 ± 0.04	0.15 ± 0.01
Pseudotsuga menziesii	18	52.8 ± 3.20	0.111 ± 0.01	1.39 ± 0.19	0.13 ± 0.04
Sophora microphylla	18	179.4 ± 28.30	0.275 ± 0.08	1.72 ± 0.15	0.13 ± 0.02
Sophora tetraptera	18	129.7 ± 17.79	0.270 ± 0.07	1.80 ± 0.09	0.22 ± 0.02
Trifolium repens	18	334.7 ± 51.51	1.464 ± 0.36	2.63 ± 0.07	0.18 ± 0.01
Ulex europaeus	18	323.6 ± 29.10	1.347 ± 0.27	2.09 ± 0.05	0.12 ± 0.01
All Fabaceae	135	285.7 ± 18.34	0.936 ± 0.10	2.10 ± 0.05	0.15 ± 0.01
All non-Fabaceae	142	199.4 ± 9.22	0.460 ± 0.05	1.37 ± 0.04	0.16 ± 0.01
All native plants	139	237.2 ± 12.74	0.549 ± 0.06	1.58 ± 0.05	0.16 ± 0.01
All exotic plants	138	245.7 ± 16.56	0.836 ± 0.10	1.83 ± 0.06	0.15 ± 0.01
All native Fabaceae	69	228.4 ± 22.00	0.491 ± 0.08	1.88 ± 0.08	0.18 ± 0.01
All exotic Fabaceae	66	345.7 ± 27.94	1.402 ± 0.18	2.31 ± 0.04	0.13 ± 0.01
All native non-Fabaceae	70	245.9 ± 13.12	0.607 ± 0.08	1.32 ± 0.05	0.15 ± 0.01
All exotic non-Fabaceae	72	154.1 ± 10.58	0.318 ± 0.05	1.42 ± 0.06	0.17 ± 0.01
All ECM plants	53	148.1 ± 15.30	0.489 ± 0.08	1.25 ± 0.08	0.14 ± 0.02
All non-ECM plants	224	263.5 ± 11.91	0.740 ± 0.07	1.82 ± 0.04	0.16 ± 0.00
All AMF plants	228	271.4 ± 11.53	0.787 ± 0.07	1.75 ± 0.04	0.16 ± 0.00
All non-AMF plants	49	101.9 ± 10.54	0.253 ± 0.04	1.49 ± 0.10	0.13 ± 0.02
Scotch broom	17	535.6 ± 60.27	2.240 ± 0.42	2.30 ± 0.06	0.12 ± 0.01
All other exotic Fabaceae (excl. broom)	49	279.8 ± 25.55	1.111 ± 0.18	2.32 ± 0.05	0.14 ± 0.01

Table 3. Linear mixed-effect model results for *C. scoparius* % coverage, plant origin, and legume status (top table) and *C. scoparius* coverage, plant origin, and ectomycorrhizal (ECM) status (below table). Accompanying *t*-values are compiled in Appendix A2. Non-significant terms only included if part of significant higher level interaction. "**.**" indicates term dropped during model simplification.

	Broom coverage	Fabaceae	Native	Broom coverage × Fabaceae	Broom coverage × Native	Fabaceae × Native	Broom coverage × Fabaceae × Native
Shoot mass (g)	**< 0.0001**	**0.0365**	0.1843	**< 0.0001**	0.0566	**0.0075**	**< 0.0001**
Root mass (g)	**< 0.0001**	0.9374	0.9760	**0.0247**	0.6540	0.0561	**0.0015**
Whole plant mass (g)	**< 0.0001**	**0.0388**	0.2879	**< 0.0001**	0.0723	**0.0092**	**< 0.0001**
Shoot N (%)	0.1327	**< 0.0001**	0.1181	0.3362	0.9227	0.3750	**0.0137**
Shoot P (%)	**.**	0.7259	0.3788	**.**	**.**	**0.0340**	**.**
Shoot N (%) / P (%)	0.6784	**0.0002**	**0.0072**	**.**	**0.0009**	**0.0497**	**.**
Total N / Total P	**< 0.0001**	**0.0007**	**0.0192**	**< 0.0001**	**0.0018**	**0.0023**	**< 0.0001**

	Broom coverage	Fabacea	Native	Broom coverage × ECM	Broom coverage × Native	ECM × Native	Broom coverage × ECM × Native
Shoot mass (g)	**< 0.0001**	0.3408	0.1987	**0.0038**	**0.0271**	**0.0463**	**0.0010**
Root mass (g)	**< 0.0001**	0.3515	0.8359	**0.0147**	**.**	**0.0467**	**.**
Whole plant mass (g)	**< 0.0001**	0.4877	0.3006	**0.0001**	**0.0045**	0.0888	**0.0225**
Shoot N (%)	0.1376	**0.0247**	**.**	**0.0073**	**.**	**.**	**.**
Shoot P (%)	**.**	**.**	**.**	**.**	**.**	**.**	**.**
Shoot N (%) / P (%)	0.6781	0.3451	**0.0408**	**0.0003**	**0.0056**	**.**	**.**
Total N / Total P	**< 0.0001**	0.1624	0.0504	**0.0037**	**0.0005**	0.1289	**0.0043**

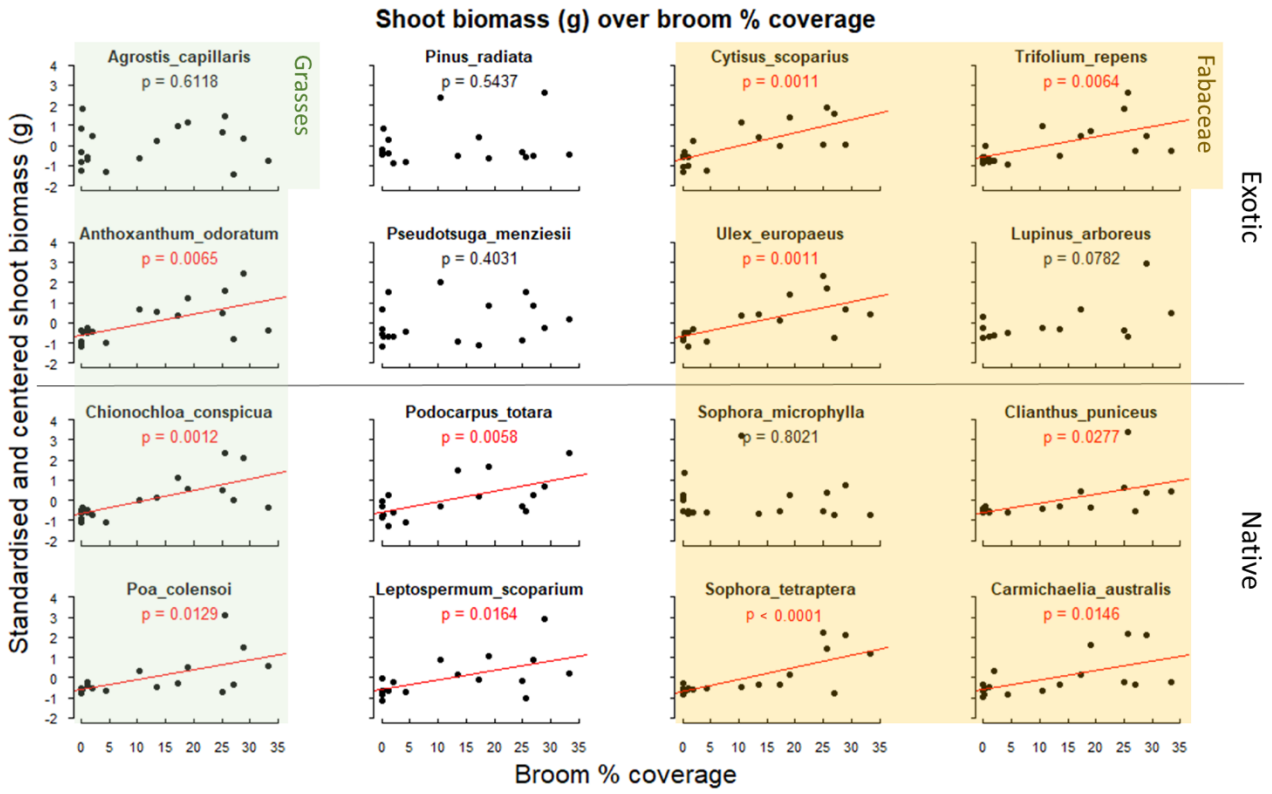


Figure 4. Standardised and centred aboveground dry biomass (g) over *C. scoparius* % coverage for all 16 plant species. Regression lines are shown when $P < 0.05$.

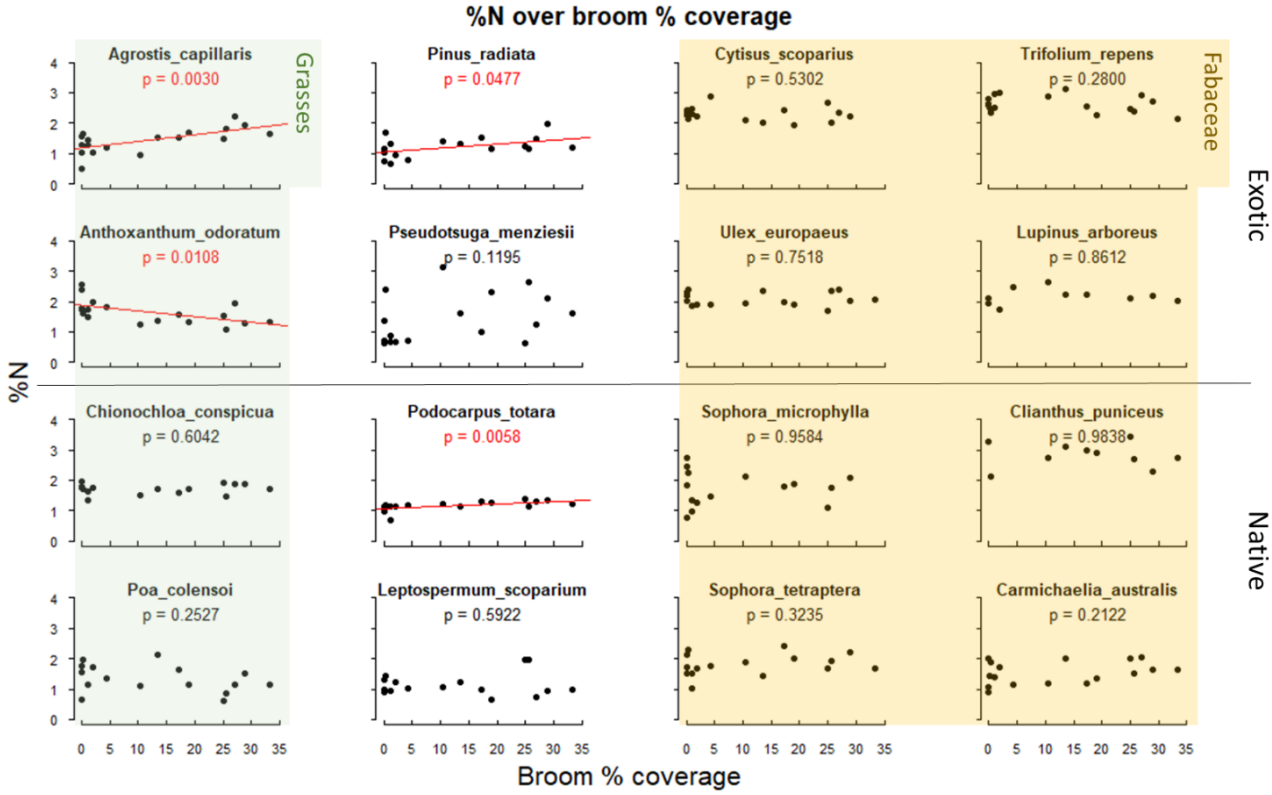


Figure 5. Shoot % N over *C. scoparius* % coverage for all 16 plant species. Regression lines are shown when $P < 0.05$.

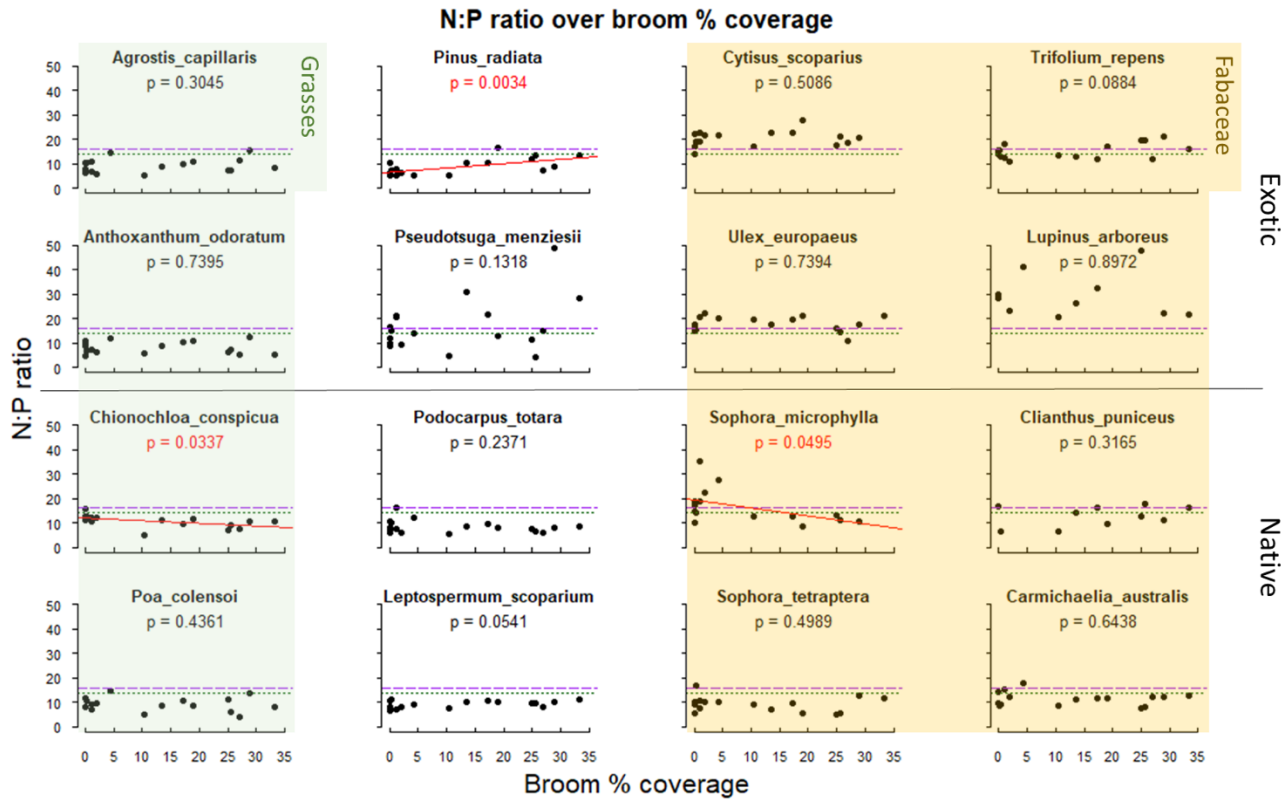


Figure 6. N:P (%) ratio over *C. scoparius* % coverage for all 16 plant species. Regression lines are shown when $P < 0.05$. Plants above the dashed purple line are putatively P-limited, whereas plants below the dotted green line are putatively N-limited (Koerselman and Meuleman 1996). Plants between both lines can be limited by either N or P or both nutrients.

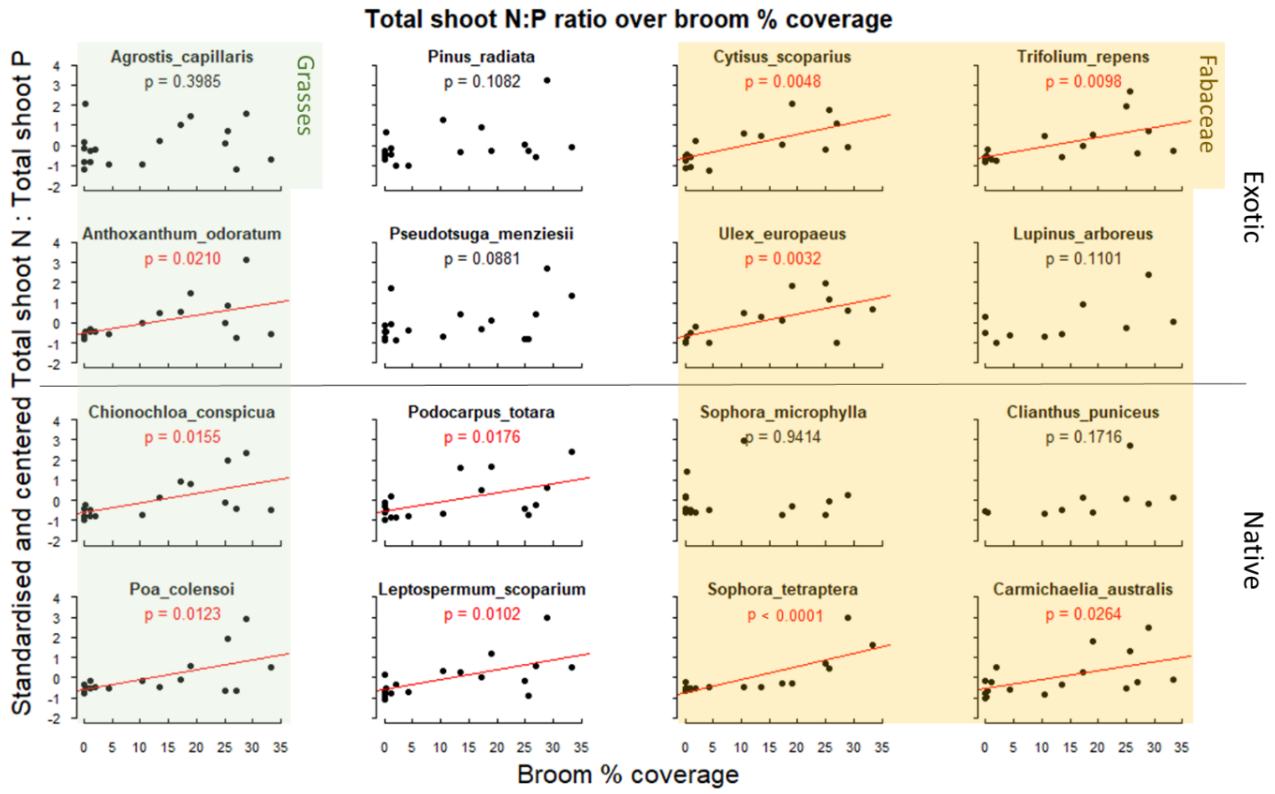


Figure 7. Standardised and centred total shoot N:P ratio over *C. scoparius* % coverage for all 16 plant species. Regression lines are shown when $P < 0.05$.

Table 4. Standardized linear coefficients of soil chemistry and plant traits correlates of plant measurements after model simplification based on *drop1* "lme4 (v1.1)" package. Dashes (-) indicate not included. All coefficients obtained from the model after *drop1* simplification are reported, regardless if significant. *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$.

		Shoot mass (g)	Root mass (g)	Whole plant mass (g)	Shoot N (%)	Shoot P (%)	Shoot N (%) / Shoot P (%)	Total N (Shoot)	Total P (Shoot)	Total N / Total P
Broom % coverage		**0.447*** **	**0.287*** **	**0.312*** **	2.418	0.427	2.557	**0.217*** **	**0.968*** **	**0.024*** **
Fabaceae		0.928	-0.270	0.865	**4.257*** **	-1.143	**3.800** **	**1.440** **	**0.730***	**1.518** **
Native		0.194	0.418	0.428	0.051	-0.904	**0.298***	0.030	-0.107	**0.111***
Soil chemistry	C (%)	_	_	_	_	_	_	_	_	_
	N (%)	_	_	_	_	_	_	_	_	_
	Olsen P (mg/kg)	_	_	_	_	_	_	_	_	_
	Ca (cmol(+)/kg)	**2.59** **	_	_	_	_	_	**2.677** **	_	**3.660*** **
	Mg (cmol(+)/kg)	**-2.087***	_	_	_	_	_	**-2.075***	_	**-3.289** **
	K (cmol(+)/kg)	_	_	_	_	_	_	_	_	_
	Na (cmol(+)/kg)	_	_	_	_	_	_	_	_	_
Broom % coverage × Fabaceae		**6.606*** **	**3.816***	**5.826*** **	-2.386	-0.722	-1.774	**7.453*** **	**5.620*** **	**7.236*** **
Broom % coverage × Native		2.111	1.977	1.680	-1.699	0.522	**-2.980*** **	**1.096***	2.049	**0.952** **
Fabaceae × Native		**-0.803***	-0.400	**-0.907***	-1.421	1.360	-1.829	**-0.860***	-0.547	**-0.982** **
Broom % coverage × Native × Fabaceae		**-4.990*** **	**-3.140** **	**-3.974*** **	**2.423***	0.923	0.723	**-4.375*** **	**-3.120** **	**-4.871*** **

Discussion

The results of this study suggest that, compared with uninvaded soil, plants grown in soil with *C. scoparius'* legacy have 1) higher above- and belowground biomass and higher total N:P ratios, particularly for native plants, 2) lower root:shoot ratios, 3) no apparent changes in % P, and 4) a mixed response concerning % N, where *C. scoparius* coverage did not change shoot % N in any tested Fabaceae, yet affected half of the non-Fabaceae. I reject my hypothesis that the soil legacy of *C. scoparius* has a more positive effect on exotic plants than on natives. Regarding my hypothesis that the soil legacy of *C. scoparius* will favour members of its own taxonomic family, I conclude that *C. scoparius'* soil legacy does not discriminate against its taxonomic family, yet does not necessarily favour non-Fabaceae either. Although some soil chemical traits had a slight correlation with the effect of *C. scoparius* coverage on plant growth, the biological effect of *C. scoparius* coverage superceded that of soil chemistry.

General effect of the soil legacy of C. scoparius on biomass

Cytisus scoparius invasion has been associated with an increase in exotic plant species and a decline in native species (Shaben and Myers 2010). For a given plant species, soil obtained from closely related plants has generally been considered to have a more negative effect on plant growth than soil obtained from distantly related plants (Kempel *et al.* 2018). My results show no strong indication of the soil legacy of *C. scoparius* having any taxonomic bias, but rather show that *C. scoparius* favours native plants over exotics. The soil legacy of *C. scoparius* increased the biomass of all but one of the eight native plant species in my experiment, compared with benefiting only half of the tested exotic species. Although the mean biomass of invasive Fabaceae outweighed that of native Fabaceae, once normalized, the effect of *C. scoparius'* soil legacy did not discriminate between native and exotic Fabaceae in terms of biomass, nor between Fabaceae and non-Fabaceae.

It is likely that unaccounted factors contributed to the shrub's dominance in-field, such as the rapid seedling growth rate of *C. scoparius* (Grotkopp and Rejmánek 2007) and the tendency of *C. scoparius* to reduce the availability of light and soil water needed by other plants (Watt *et al.* 2003b, Wearne and Morgan 2004). We found in a field experiment that plant growth next to live *C. scoparius* was most beneficial for exotic legumes compared to native legumes (Allen *et al.* (2020); Appendix E), yet especially when the rhizosphere of *C. scoparius* was allowed to influence plant growth. I can therefore speculate that the belowground association of *C. scoparius* with soil organisms (Grove *et al.* 2017) is another factor contributing to the dominance of *C. scoparius* in-field and may underlie the results of my soil legacy experiment. One possible reason as to why field-based studies on the

performance of native NZ plants showed the negative responses to *C. scoparius* invasion (e.g. Allen *et al.* 1995; Watt *et al*. 2003) is that native plants still need to deal with co-evolved antagonists in situ (e.g. specialist pathogens). A possible consideration would be that beneficial effects of mutualists associating with *C. scoparius* may have shifted to the native plants, thereby counterbalancing the antagonists.

Plant root:shoot ratios are likely to decline when conditions for growth improve as a result of increased soil moisture or nutrient availability (Wilson 1988). As moisture availability was uniform for all plants due to growing in greenhouse conditions, seeing a decline in root:shoot ratio, which was most apparent for two native species, suggests that *C. scoparius* increases soil nutrient availability.

Plant specific effects of C. scoparius soil legacy on biomass

It was expected that shoot biomass did not increase with *C. scoparius* coverage for *Pseudotsuga menziesii* as negative effects of *C. scoparius* on *P. menziesii*, driven largely by reduced ectomycorrhizal fungal colonization, have been documented (Grove *et al.* 2012). As *Pinus radiata* is also primarily ectomycorrhizal (Teste *et al.* 2020), finding no increase in biomass with *C. scoparius* coverage was also expected and could broadly be attributed to the need for ectomycorrhizal seedlings to have appropriate mycorrhizal inoculum for effective growth (Lekberg and Koide 2005, Dickie *et al.* 2012).

It was less expected to find an increase in the biomass of native ectomycorrhizal *Leptospermum scoparium* (mānuka), which likewise had to contest with a probable reduction in available ectomycorrhizal inoculum. However, among native New Zealand plants which form ectomycorrhizal associations, *L. scoparium* is known to associate with both arbuscular mycorrhizal and ectomycorrhizal fungi (Davis *et al.* 2013). Being dual-mycorrhizal, *L. scoparium* could have opted into associating with arbuscular mycorrhizal fungi (Teste *et al.* 2020), which could partially explain why *L. scoparium* was not hindered in its growth as *C. scoparius* coverage increased.

Effect of C. scoparius soil legacy on % N and % P

Based on nutrient limitation indicators proposed by Koerselman and Meuleman (1996), where plant N:P ratios >16 indicate putative P limitation and plant N:P ratios <14 indicate putative N limitation, it can be observed that exotic Fabaceae are prone to being P-limited whereas all other plants, particularly the grasses and native non-Fabaceae, are N-limited. *Sophora microphylla* was here a unique case in that increased *C. scoparius* coverage seemed to transition the plant from a state of putative P limitation to putative N limitation, however this transition was not observed for other native Fabaceae.

The direction of changes in shoot % N did not necessarily correlate with corresponding changes in shoot biomass. As live *C. scoparius* presence has been associated with an increase in the cover of exotic *Anthoxanthum odoratum* (Carter *et al.* 2019c), seeing an increase in the aboveground biomass of *A. odoratum* with *C. scoparius* coverage was expected, yet not alongside a decrease in aboveground % N. On the other hand, the aboveground biomass of both *Pinus radiata* and *Agrostis capillaris* remained unchanged with *C. scoparius* coverage, even though both plants showed increases in shoot % N over *C. scoparius* coverage. Although Watt *et al.* (2003b) had shown that juvenile *Pinus radiata* grew markedly less alongside live *C. scoparius*, the same authors suggested that *C. scoparius* may in the long-term enhance the growth of *Pinus radiata* in N-deficient sites, provided that water stress did not impair nutrient uptake (Nambiar and Sands 1993). Observing an increase in the shoot % N of *Pinus radiata* without seeing any change in shoot biomass over *C. scoparius* coverage could be attributed to pine in general having specific growth spurts even if unrestricted by available N (Fagerström and Lohm 1977). Regarding *A. odoratum*'s decrease in % N despite an increase in aboveground biomass, *A. odoratum* is known to be a relatively fast growing grass which accumulates large quantities of soil N compared with slower growing grasses (Weigelt *et al.* 2005). The simplest explanation to *A. odoratum's* decrease in % N is that the grass is responding to some other limiting resource, possibly soil C.

Effect of C. scoparius coverage on soil chemistry

Although *C. scoparius* is known to increase soil organic C (Fogarty and Facelli 1999), the availability of soil P (Dewar *et al.* 2006) (although see Shaben and Myers (2010)), as well as soil N (Watt *et al.* 2003b), there was no correlation observed between *C. scoparius* coverage and soil chemistry in my data. Broadbent *et al.* (2017), who used the same data on soil properties yet separate data on *C. scoparius* coverage, also reported that *C. scoparius* coverage frequently led to no significant difference in soil properties. Caldwell (2006) observed that soil under *C. scoparius* had a significantly higher C:P ratio than soil from an adjacent area uninvaded by *C. scoparius*, yet I found no significantly different C:P ratio in my data. Finding no correlation between *C. scoparius* coverage and soil chemical attributes was least expected for soil N, as soil N has often been documented to increase following a *C. scoparius* invasion (Haubensak and Parker 2004, Caldwell 2006, Grove *et al.* 2015), although no changes in soil N caused by *C. scoparius* have also been reported (Shaben and Myers 2010, Carter *et al.* 2019c). Change in soil N under *C. scoparius* has been described as site-specific (Slesak *et al.* 2016), which could be the case in my data.

It has been suggested that the competitive ability of *C. scoparius* correlates with nutrient availability (Fogarty and Facelli 1999). As an explanation as to why no correlation between soil nutrients and *C. scoparius* coverage was found in this experiment, it could be proposed that *C. scoparius* (or other plants in the field) had already taken up any additional available nutrients which *C. scoparius* may

have imparted on the soil or, more simply, that no significant changes in soil chemical composition were caused by *C. scoparius*.

Experimental design considerations

A common issue with soil legacy studies is that it is difficult to separate the chemical, physical and biological properties in a given soil which can each play differing roles in determining plant growth (Van der Putten *et al.* 2013). Biological properties can be particularly underrepresented: certain plants exert a disproportionately great influence on soil biota (Weir *et al.* 2004), even with a small biomass relative to other plants (Peltzer *et al.* 2009). In contrast, the effect of certain biochemical plant properties might have been overrepresented: *C. scoparius* has been considered as putatively allelopathic in a study by Grove *et al.* (2012), who amended soils from four different sites with sucrose, activated C and *C. scoparius* litter and examined the effect of *C. scoparius'* soil legacy on a the growth of a single pine species (*Pseudotsuga menziesii*). Allelopathy has been a contentious topic since its conception (Williamson 1990) (but see Bais *et al.* (2003), Pardo-Muras *et al.* (2020)) and my observed overall increase in plant biomass over *C. scoparius* coverage gives no indication of allelopathy. The effect of *C. scoparius* on soil N has already been deemed site-specific (Slesak *et al.* 2016) and it may be proposed that effects of *C. scoparius'* soil legacy attributed to allelopathic compounds might in fact be due to the shrub's association with certain site-specific micro-organisms.

Although no obvious correlation has been observed in my dataset (Appendix A6), soil under a *C. scoparius* invasion in New Zealand has been found to have significantly higher available P than uninvaded native soil (Dewar *et al.* 2006). There is some uncertainty as to whether *C. scoparius* caused an increase in available P or vice-versa, as superphosphate fertilizers with long-lasting effects have been applied to large areas of New Zealand by aerial means (During 1972, Sharpley and Syers 1979, Will *et al.* 1985). Caution is needed when drawing conclusions related to soil nutrients, as these are known to vary between seasons (Powers 1990, Gilliam *et al.* 2001), yet my soil nutrient data (used by Broadbent *et al.* (2017)) should still reliably reflect *C. scoparius* presence as no recent change in *C. scoparius* invasion to the Molesworth plots was observed.

It is notable that native grasses grown in competition with *C. scoparius* have been shown to inhibit *C. scoparius* development (Harrington 2011, Lang *et al.* 2017), and the Molesworth field site used in this experiment does have near mono-dominant patches of *C. scoparius* in close proximity to scarcely disturbed grassland. I cannot rule out the possibility that the soil legacy of the native grasses decreased plant biomass (as opposed to *C. scoparius'* soil legacy being solely responsible for the observed trends). A future research direction would be to include sterilized soil treatments when performing a similar experiment to account for the putative effects of such native grasses or other biotic variables.

Conclusions and applications

Although in-field observations imply the opposite, the soil legacy of *C. scoparius* unexpectedly favours the growth of native New Zealand plants over its own taxonomic family. Given that *C. scoparius'* predominantly positive soil legacy effect can only be partly attributed to soil chemical traits, microbial effects could very well play an important role in the invasion success of *C. scoparius* (Haubensak and Parker 2004). Compared with classic soil legacy studies, much less is known on the importance of soil legacies involving possible co-invading belowground mutualists (Nuñez and Dickie 2014). My next chapter will delve deeper into the effect of *C. scoparius* on surrounding microbial composition, specifically that of soil fungi.

Chapter 3: The response of fungal communities to *Cytisus scoparius* invasion

Abstract

Homogenization caused by introduced plants typically results in decreases in the species richness of associating organisms, at least at the local scale. Although studies on the response of communities to a plant invasion have often taken an aboveground focus, less is known regarding the belowground consequences of a plant invasion, despite soil communities being integral to conservation efforts and having a long-lasting effect on plant community composition. To study the effect on an invasive nitrogen-fixing plant on soil fungal communities, I examined metabarcoded environmental DNA data from soil across a natural *Cytisus scoparius* density gradient. I categorised fungal operational taxonomic units into specific functional guilds such as saprotrophs and plant pathogens, which allowed a more thorough examination of how *C. scoparius* affected fungal communities. Although I found that certain fungal groups became more homogeneous under higher densities of *C. scoparius* (likely caused by increased plant homogeneity), my results showed that *C. scoparius* invasion increased average fungal diversity at the point-scale per plot (likely caused by increased soil productivity). A greater proportion of unique fungal taxonomic units were found near *C. scoparius* as opposed to uninvaded grassland and soil under *C. scoparius* had a higher richness of saprotrophs, plant pathogens and arbuscular mycorrhizal fungi. Despite leading to a lower plant diversity, it is possible that changes in soil properties induced by *C. scoparius*, such as increased N deposition and water retention, enabled a higher richness of fungi to live alongside the invasive shrub. My results indicate that coalescence between previously separated fungal communities may have occurred due to *C. scoparius*. Apart from *C. scoparius* having a definite effect on soil fungal communities, it is possible that the soil fungal communities themselves might contribute to the shrub's invasiveness.

Keywords

Arbuscular mycorrhizal fungi, diversity, environmental DNA, soil communities, metabarcoding

Introduction

Soil biodiversity patterns are moulded by a wide range of (a)biotic factors which operate at different spatial scales (Ettema and Wardle 2002). At the smallest spatial scale (micrometre to millimetre), distribution patterns of soil biota are in part affected by rooting patterns in plants and microscale soil heterogeneity (Bardgett and Van Der Putten 2014). At a fine-scale (millimetre to centimetres), spatial patterns in microbial communities are influenced to some extent by root exudates (Broeckling *et al.* 2008), which attract microbial symbionts to roots, including rhizobia, mycorrhizal fungi (Badri and Vivanco 2009), and soil pathogens (Mendes *et al.* 2011). Chemical and physical soil properties (e.g., soil nutrient availability and soil water) alongside the identity of dominant plants determine spatial patterns of soil biota at the local scale (centimetres to metres) (Wardle 2013). At even larger regional and continental scales, which can range from metres to hundreds of kilometres, dynamics such as topography, climate and continental isolation play a more important role (Fierer and Jackson 2006).

Plant-mediated changes to soil microbial composition at the local scale (centimetres to metres) are virtually omnipresent in natural environments, as soil micro-organisms form symbiotic interactions with most plants (Smith and Read 2010) and these plants can have a profound influence on the structure of surrounding microbial communities (Tedersoo *et al.* 2016, Kivlin *et al.* 2018). Most research on a plant's effect on soil communities has taken a plant-soil feedback approach, measuring plant growth responses but not identifying species of microbes (Bever *et al.* 2010). In spite of an increase in plant-soil feedback studies, relatively little is known about how plants modify soil microbial community composition (Eisenhauer *et al.* 2010, Maul and Drinkwater 2010). Even at low biomass, some plants are capable of "punching above their weight" in terms of modifying soil properties (Peltzer *et al.* 2009) and invasive plants can cause changes in soil microbial communities with long-lasting consequences to ecosystem function (Nuñez and Dickie 2014). The abundance, activity and composition of soil microbial communities have long been known to vary with different plant species (Bever *et al.* 1997), yet further than just passively affecting microbial communities in their surrounding soil, plants have been known to actively cultivate their own associating micro-organisms (Broz *et al.* 2007, Broeckling *et al.* 2008). Such affiliations between plants and belowground mutualists can be very specific (Stefanowicz *et al.* 2019), with the spread of certain plants having been linked with a single species of associating micro-organism (Hayward *et al.* 2015). Both the plant and the plant's mutualists have become invasive in certain cases (Marler *et al.* 1999, Simberloff and Von Holle 1999, Richardson *et al.* 2000, Callaway *et al.* 2001), with profound consequences on ecosystem development (Dickie *et al.* 2019).

Given the numerous spatial scales at which soil biota associated with a plant invasion can be studied, there is limited information on how an invasive plant can change belowground microbial diversity and heterogeneity across different spatial scales. Although changes in soil community composition have been analysed in successional studies (Van der Putten *et al.* 1993, Dickie *et al.* 2019) and across environmental and latitudinal gradients (Sharp *et al.* 2014, Tedersoo *et al.* 2014, Lu *et al.* 2018), the belowground consequences of invasive plants on soil biota remain less known (Van der Putten *et al.* 2007b).

There is generally a positive relationship between the biodiversity of groups of directly or indirectly interacting organisms (Gaston 2000, Scherber *et al.* 2010, Peng *et al.* 2019), particularly in terms of beta diversity. As such, it is expected that increased plant diversity correlates with increased belowground fungal diversity, as has been shown for ectomycorrhizal fungi (Dickie 2007, Lang *et al.* 2011, Gao *et al.* 2013). The richness of arbuscular mycorrhizal fungi (AMF) is also positively associated with plant species richness (Vogelsang *et al.* 2006, Hiiesalu *et al.* 2014), although no association between plant richness and AMF richness has likewise been recorded (Öpik *et al.* 2008, Lekberg *et al.* 2013). It is known that a plant invasion can actually increase both the abundance and diversity of AMF compared to native uninvaded soil (Lekberg *et al.* 2013).

While decreases in local diversity caused by an invasive plant are typical (Hejda *et al.* 2009, Powell *et al.* 2011, Lishawa *et al.* 2019), plant-induced increases in local diversity have been related to improved ecosystem productivity (Liao *et al.* 2008, Ehrenfeld 2010). Nutrient cycling commonly underlies ecosystem productivity (Ehrenfeld 2003, Daryanto *et al.* 2019) and may be enhanced by interactions between plants and associated AMF (Saia *et al.* 2020b) and/or N-fixing Rhizobia. Although a meta-analysis on the effects of both alien N-fixing plants and alien non-N-fixing plants has shown similar degrees of impact on native plant communities (Vilà *et al.* 2011), this pattern does not address belowground soil biota. Changes in vegetation caused by N-fixers can have a very long-lasting effect on N availability and nutrient cycling (Hu *et al.* 2001), which may in turn affect the composition of soil biota. Simulated N deposition has been correlated with decreases in AMF root colonization and spore production (Van Diepen *et al.* 2011), alongside decreases in AMF diversity (Wang *et al.* 2011, Lin *et al.* 2012), ectomycorrhizal fungi diversity (Wright *et al.* 2009) and general soil fungal diversity (Edwards *et al.* 2011, Paungfoo-Lonhienne *et al.* 2015, Zhou *et al.* 2016). N deposition also has a potential to negatively impact on C cycling in soil and may promote fungi which have pathogenic traits (Paungfoo-Lonhienne *et al.* 2015). Although decreases in fungal diversity are most common following N deposition, outcomes may vary according to the studied system as N fertilizer has been shown to both increase (Klaubauf *et al.* 2010) and decrease (Edwards *et al.* 2011) the relative abundance of the fungal phylum Ascomycota (the most common fungal phylum in both studies).

Most studies which focus on the effect of invasive plants on soil fungal communities use experimental approaches rather than undertaking a natural survey. For example, Gornish *et al.* (2016) simulated different levels of plant invasion by seeding plots of *Taeniatherum caputmedusae*, as opposed to sampling across a natural invasion gradient. Burke *et al.* (2019) and Anthony *et al.* (2019), who both studied the effect of invasive *Alliaria petiolate* on fungal communities, used exclosure and control plots and an eradication-based experimental design, respectively.

How soil communities are identified and categorised may also be an important factors. Gaggini *et al.* (2018) found that invasive *Impatiens glandulifera* increased soil fungal community diversity. In a study on how three exotic grasses added to experimentally grown native monocultures modified soil microbial composition, Gibbons *et al.* (2017) found no significant changes in fungal alpha and beta diversity caused by any of their studied invasive grasses. However in the same study, when fungal operational taxonomic units (OTUs) were assigned to functional guilds, two exotic grasses caused a decrease in fungal OTUs assigned as symbionts and one exotic grass caused an increase in fungal OTUs assigned as pathogens, whereas none of the three tested grasses caused a change in the richness of saprotrophic fungi (i.e., decomposers).

At a broader scale, fungal species identified as saprotrophs are known to have an increased abundance in native forests compared to forests invaded by *Ligustrum lucidum* (Fernandez *et al.* 2017) and fungal species diversity has generally been found to decrease with increased density of invasive plants such as *Centaurea maculosa* (Broz *et al.* 2007), *Ageratina adenophora* (Balami *et al.* 2017) and *Robinia pseudoacacia* (Liu *et al.* 2018), although an increase in fungal richness was observed for *Alliaria petiolata* (Anthony *et al.* 2017). All the above studies either use artificially created monocultures or a direct "control vs. effect" experimental design instead of sampling across an in-situ gradient of plant invasion. Moreover, none of the studied plants in these studies, apart from *Robinia pseudoacacia* (Liu *et al.* 2018), are N-fixers.

There is little known regarding the effect of my species of interest, *C. scoparius*, on microbial communities (although see Johnston *et al*. 1995). Compared with uninvaded soil, *C. scoparius* increases microbial biomass N, microbial biomass P and microbial biomass C (Dewar *et al.* 2006), hence a case can be made that the proportional composition of certain fungal taxa might increase following a *C. scoparius* invasion.

I chose to focus on soil fungi as they have a better "species concept" than bacteria, in part as functional genes are less frequently horizontally transferred between fungal species than in bacteria (Klingmüller *et al.* 1990). Soil fungi have also been observed as being more spatially heterogeneously distributed in soil compared to bacteria (Manter *et al.* 2010), and are more responsive to the identity of specific invasive plants (Maron *et al.* 2011, Xiao *et al.* 2014, Bahram *et al.* 2020). With regard to plant pathogens, most plant pathogen OTUs across different landscapes

in New Zealand have been identified as fungi as opposed to bacteria or oomycetes (Makiola *et al.* 2019a). Studying fungal OTUs is hence likely to give an accurate depiction of how an invasive plant modifies soil pathogen communities, especially as fungal pathogens are more intimately associated with living plants compared to most bacteria (Webster and Weber 2007, Bahram *et al.* 2020). With regard to fungal saprotrophs, these have also been known to outnumber bacteria within the decomposer community (Maraun and Scheu 1996).

Using spatially explicit data on fungi collected across the density gradient of an invasive plant, my aim was to survey how soil fungal communities responded to a *C. scoparius* invasion. Seeing near-monocultures of invasive plants is an obvious indicator of decreased plant diversity and increased plant homogeneity (Waterhouse 1988), which should accompany a decrease in the diversity of directly or indirectly associating organisms (Gaston 2000, Scherber *et al.* 2010). Anything contributing to N input in an ecosystem, such as *C. scoparius'* association with N-fixing rhizobia, is also likely to increase homogenization (Olden 2006). Here I look at three levels of diversity. I use "gamma diversity" as the number of fungal species found in a 20 × 20 m plot, and alpha diversity as the number of fungal species found in a soil core. I measure beta diversity (fungal community heterogeneity) as the ratio between plot level gamma diversity and soil core level alpha diversity (Whittaker 1970).

I hypothesised that:

- *C. scoparius* invasion will result in a decrease in soil fungal gamma diversity and alpha diversity.
- *C. scoparius* invasion will result in a decrease in soil fungal heterogeneity (beta diversity) within 20 × 20 m plots.
- Given the mostly positive soil legacy of *C. scoparius* observed in my soil legacy experiment (Chapter 2), that soil invaded by *C. scoparius* will contain fewer antagonists.
- Given that *C. scoparius* has been known to increase microbial biomass, that soil invaded by *C. scoparius* will undergo shifts in the proportional abundance of certain taxa and/or functional groups.

In addition to the hypotheses, I also investigated how *C. scoparius* might affect fungal community composition and OTU occupancy across plots, enabling a more comprehensive view of belowground changes which correlate with *C. scoparius* coverage. Metabarcoding is well suited to deal with the diversity and identity of soil fungi in novel ecosystems (Dickie and St John 2016) and has recently been successfully applied to study large-scale patterns in the distribution of soil biota (Makiola *et al.* 2019a). To test these hypotheses, I examine metabarcoded eDNA data from

432 georeferenced soil cores extracted from 18 plots (24 soil extracts per plot) across a natural *C. scoparius* density gradient. I categorised fungal OTUs into specific functional guilds such as saprotrophs and antagonists, which allowed a more thorough examination of *C. scoparius'* effect on co-occurring fungi (Zanne *et al.* 2019).

Methods

Study site and natural experiment

The study site was located in the Saint James conservation area in New Zealand's South Island (-42.460273 Lat., 172.830938 Long.; elevation = 800–900 m.a.s.l.; mean annual temperature = 10.3°C; mean annual rainfall = 1158 mm, Hanmer forest weather station) (Figure 1). *Cytisus scoparius* (Scotch broom) is widely spread throughout this region and a description of the site's vegetation is given in Broadbent *et al.* (2017). Permanent 20 × 20 m vegetation plots were laid out at the site by Manaaki Whenua – Landcare Research, following standard field protocols in Hurst and Allen (1993). For this experiment, I selected 18 permanent vegetation plots across a *C. scoparius* density gradient (including 3 plots without any *C. scoparius* and 15 plots from low to high *C. scoparius* density) and all plots were located within 2.5 km of each other. These 18 plots were the same used for soil extraction in my soil legacy experiment (Chapter 2).

I undertook field sampling from 14 February 2017 to 17 April 2017. For each of the 18 permanent vegetation plots, I extracted 24 individual georeferenced soil cores (Figure 2), for a total of 432 spatially explicit soil samples. To reliably pinpoint areas for soil extraction, I subdivided each plot into 5 × 5 m subplots by laying out measuring tapes at 5 m intervals. All extractions were taken at their precise planned location and no sampling deflection (due to immovable objects, e.g., tree trunks) was necessary. I kept a catalogue of natural occurrences which might affect the composition of extracted soil (e.g., soil was dug up from within an active ant-nest and beneath a decomposing hare carcass) and I recorded the following *C. scoparius* coverage measurements at each point of soil extraction: 1) distance to closest immature *C. scoparius*, 2) distance to closest mature *C. scoparius* (determined by the presence of flowers or seedpods), 3) height of the tallest *C. scoparius* within 1 m radius, and 4) a *C. scoparius* density estimate within 1 m radius, which I refer to as "broom coverage".

Prior to soil extraction, metal trowels were manually scrubbed and then sterilized in a 10% v/v bleach solution (8% sodium hypochlorite in undiluted bleach) for >10 min (Prince and Andrus 1992) before being rinsed in water. I dug up soil samples vertically to a depth of 150-200 mm, each soil sample weighing 200–250 g. Litter and leaf matter, typically forming the top 10-20 mm of a soil sample, was discarded. Once dug up, soil samples were sealed in individual, clean, zip-lock plastic bags and transported in insulated ice chests prior to being stored at 4°C until subsequent processing. The time between field extraction and refrigeration at 4°C was restricted to a maximum of 60 hours and the soil samples were left refrigerated until further processing for a maximum of 14 days. The collected samples were stored in a fridge temporarily, as opposed to a freezer, as the

soil would otherwise be subject to unwanted freeze-thaw cycles, which disturbs microbial communities (Pesaro *et al.* 2003). Each refrigerated soil sample was broken up manually and spread out evenly on clean paper. Using bleached forceps and spatulas, I obtained a ~10 g mixed soil sample by systematically extracting 10 × ~1 g subsamples from across the initial sample. The mixed subsampled soil did not contain any roots more than 5 mm in width or stones larger than 5 mm in diameter and obvious insects (e.g., ants, larvae) were avoided. I kept the processed soils frozen at -18°C until DNA extraction.

Wet-lab processing

Both the kit used for soil DNA extraction and the chosen fungal primers were recommended by Lear *et al.* (2018). I performed DNA extraction on the 432 soil cores using DNeasy PowerSoil® HTP 96 Kits (Quiagen), according to the manufacturer's instructions and loading the maximum amount of recommended soil for DNA extraction (250 mg). As part of the PowerSoil® protocol, mechanical lysis of the soil samples was performed using a Spex® Sample Prep 1600 MiniG. Based on amplification protocols outlined by the Earth Microbiome Project (Gilbert *et al.* 2014) (http://press.igsb.anl.gov/ earthmicrobiome/protocols-and-standards/its/), I assembled two single-indexed DNA libraries from the 432 soil extracts using the fITS7 general fungal primer (5′-GTG ART CAT CGA ATC TTT G -3′) (Ihrmark *et al.* 2012) and the ITS4 reverse primer (5'- TCC GCT TAT TGA TAT GC -3') (White *et al.* 1990). The ITS4 reverse primer was designed with both Illumina adapter sequences and index sequences (Caporaso *et al.* 2011), permitting future identification of the sequenced amplicons. I ordered the fITS7 primer (along with its Illumina adapter sequence) from Integrated DNA Technologies (Purification method: Standard Desalting). The Illumina adapter for fITS7 was 5'- AAT GAT ACG GCG ACC GAG ATC TAC AC -3′ and the Illumina adapter for ITS4 was 5'- CAA GCA GAA GAC GGC ATA CGA GAT -3′.

Using an Eppendorf vapoprotect Mastercycler®, PCR amplifications were performed in a 25 µL mixture volume containing 0.2 µL FastStart™ DNA polymerase (Merck), 0.5 µL dNTP mixture (10 mM each), 2.5 µL PCR buffer (with 20 mM $MgCl_2$, sourced from Merck), 2 µL 2.5 µM of each forward and reverse primer, 1.25 µL 10 µM molecular grade Bovine Serum Albumin, 1 µL 10× diluted DNA template and 15.55 µL filtered deionized water (obtained via a Milli-Q® water purification system and filtered through a Biopak® Polisher). I used Bovine Serum Albumin to reduce the effect of PCR inhibitors derived from soil (Jiang *et al.* 2005) and assembled all PCR reagents prior to adding the DNA template in a dedicated UV light irradiated chamber with a dedicated set of micropipettes. PCRs were carried out under the following conditions: a denaturation step of 5 min at 94°C, followed by 35 cycles of 30 s at 94°C, 30 s at 57°C and 30 s at 72°C, with a final step at 72°C for 7 min (and held at 4°C). All PCRs were carried out in duplicate along with positive and negative controls. To confirm amplification, I performed agarose gel

electrophoresis on the PCR product, stained with RedSafe™ (iNtRON) and using a 1% agarose gel. No PCR products were observed in negative controls and samples which showed poor amplification were rerun. The 10× dilution of the DNA templates (with filtered deionized water) was undertaken after initially performing PCRs with 1 µL of the undiluted PowerSoil® DNA elution, which yielded less PCR product according to gel runs, possibly due to PCR inhibition caused by soil components such as tannins (Kreader 1996). As the PCRs were performed in duplicate, 20 µL of each duplicate PCR product were combined prior to normalization.

Following manufacturer's instructions, I used SequalPrep™ Normalization Plates (ThermoFisher Scientific) to both clean the obtained amplicons and to normalize their concentration relative to each other. Twenty-five µL of mixed duplicate PCR product (25 µL being the recommended maximum) underwent normalization as part of the SequalPrep™ protocol. I eluted my normalized PCR product with 12 µL elution buffer (instead of 20 µL recommended by the manufacturer) due to a previous unsuccessful sequencing library submission that might possibly have been caused by too low a concentration of PCR amplicons. The resulting concentration of normalized samples was 2.14 ng/µL. I prepared two sequencing libraries; for each library, 12 µL of normalized indexed PCR amplicons were pooled together, vortexed and 110 µL each of the two resulting mixtures were sent to Massey Genome Service, New Zealand, to undergo Illumina MiSeq™ (2×250 base PE v2) (Caporaso *et al.* 2012).

Bioinformatics and statistical analysis

I merged forward and reverse Illumina reads using a 32-bit version of USEARCH v11.0.667 (Edgar 2010). I removed any sequences with less than 200bp or which had more than one expected error using VSEARCH 2.10.4 (Rognes *et al.* 2016). In order to increase the qualitative nature of the sequencing reads and to account for PCR and sequencing artefacts (Leray and Knowlton 2017) and singletons (Dickie 2010), any sequences occurring either once or twice were removed, while the remaining sequences were clustered to 97% similarity threshold. Although both USEARCH and VSEARCH could have been used to filter sequences, the 64-bit version of VSEARCH is open-source and was therefore chosen. OTUs were matched using BLAST v2.5.0+ (Altschul *et al.* 1997) against the UNITE public database (accessed July 2019) (Nilsson *et al.* 2018). I removed all recorded OTUs which were not within the kingdom Fungi and all OTUs which had a <200 bp match to any known species. Extraction blanks, and positive and negative controls were checked for contamination and OTUs which were found within my negative controls (0.34% of all OTUs) were also deleted. In order to further limit the effects of PCR and sequencing artefacts (Vesty *et al.* 2017), I excluded low abundance OTUs by setting OTU occurrence to 0 if any OTU occurred less than 3 times in any sample. In the case where the same soil extract was sent to be sequenced twice

(due to an insufficient amount of amplification observed via gel runs), the sample with the lowest number of reads was deleted along with any sample which had <1000 reads (0.23% of samples).

In order to classify fungal OTUs according to functional guild, OTUs were matched against the FUNGuild database (Nguyen *et al.* 2016). FUNGuild has shown previous effective use in identifying putative soil symbiotrophs, saprotrophs and pathogens (e.g., Gibbons *et al.* (2017), Lu *et al.* (2018)). Some fungi had multiple ecosystem functions and as many OTU classifications listed by FUNGuild overlapped with each other (e.g., an OTU could be described as both a saprotroph and a pathogen), I created two databases, one in which each OTU was strictly classified according to a single functional guild, and one in which OTUs were loosely classified and could overlap between guilds. As an example, a 'strictly' classified OTU would only be described by FUNGuild as being a saprotroph or a pathogen (and never both), whereas a 'loosely' classified OTU could be classified as both a saprotroph and a pathogen. Regarding putative pathogens in both 'strictly' and 'loosely' categorised databases, further subdivisions were created for each database: 1) a group in which all known antagonist OTUs were compiled, 2) a group specifically for antagonists of fungi and 3) a group specifically for plant antagonists.

I used R version 3.5.0 (Team 2013) for creating graphs and conducting analyses. All diversity estimates were based on evenly rarefied OTU matrices and the subsample size for rarefying my community was set to the minimum number of sequences in any sample. When examining the composition of my samples according to fungal taxon and functional guild, I first generated randomly rarefied community versions of my dataset via the *rrarefy* function in "vegan" (Oksanen *et al.* 2013). This process was iterated 250 times before calculating average alpha, beta and gamma diversities and proportional abundances for each fungal taxon and functional guild across all iterations. The *rrarefy* function was here appropriate as random rarefaction is performed without replacement so that the variance of community metrics is not related to the size of the sample. When calculating beta (β) diversity for each plot I used the below formula for true beta diversity from Whittaker (1970), where gamma (γ) is a the total species diversity in any given plot and alpha (α) is the average diversity across all soil cores (min. 23) per plot.

$$\beta = \frac{\gamma}{\alpha}$$

True beta diversity has been used in large-scale fungal studies (e.g., Kivlin *et al.* 2011). To quantify the effects of *C. scoparius* coverage on fungal alpha diversity and proportional abundance at the level of soil cores, I used linear mixed-effect models via the R package "lme4 (v1.121)" (Bates *et al.* 2014), setting sampling plot as a random effect. The mixed models break down the variance into inter- and intraplot components and thus improve the true structure of the randomness present in the data (Millar and Anderson 2004).

Figure 1. Picture of the Molesworth field site taken in March 2017 and prominently featuring a *C. scoparius* (Scotch broom) invasion.

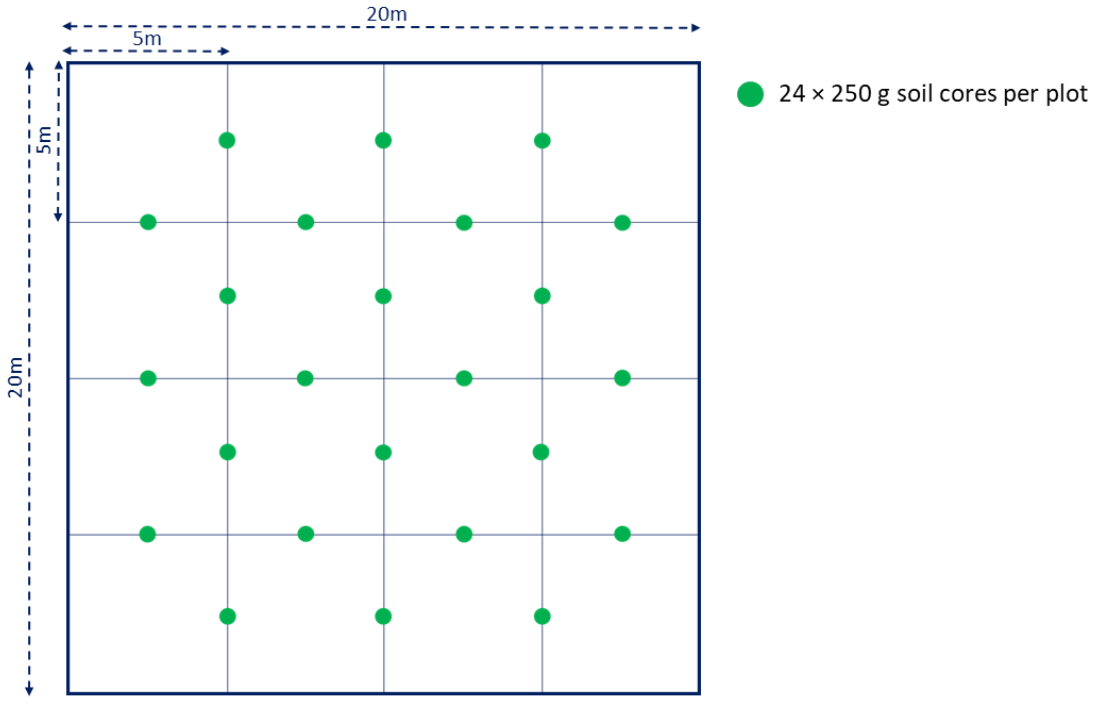


Figure 2. Overview of soil core collection from each of 18 field plots (total soil cores = 432).

Results

In total, 5263 fungal OTUs were identified from 431 soil extractions across 18 plots, the three most dominant fungal phyla and subphyla being Ascomycota (3078 OTUs; 58.5%), Basidiomycota (1606 OTUs; 30.5%) and Mortierellomycotina (152 OTUs; 2.9%) (Appendix B1). Eight out of the ten most abundant OTUs belonged to Ascomycota. A few OTUs dominated the community, with around half (50.9%) of reads belonging to the 60 most abundant OTUs (1.1% of all OTUs). Rare OTUs were common, with 57.2% of OTUs (n = 3008) occurring only between three to ten times across all samples (three being the decided minimum for any soil sample). In functional assignments, 29% of OTUs were identified as 'strict' saprotrophs, 13.9% as 'strict' symbiotrophs and 8.1% as 'strict' antagonists; but most OTUs were classified as having multiple ecosystem functions.

Overall gamma (γ) diversity

There was no significant change in gamma diversity over *C. scoparius* coverage for all fungal OTUs, nor for any individual fungal taxon or functional guild apart from Glomeromycotina (R^2 = 0.59; P < 0.0001) and Mucoromycotina (R^2 = 0.64; P = 0.0001; Appendix B2), which both showed increases in gamma diversity over *C. scoparius* coverage (Figure 3). Although no response in gamma diversity over *C. scoparius* coverage was observed for Ascomycota, the most abundant taxon across all plots, the gamma diversity of Ascomycota relative to the gamma diversity of all fungal OTUs decreased over *C. scoparius* coverage (R^2 = 0.37; P = 0.0075; Appendix B2).

Overall alpha (α) diversity

For all fungal OTUs, there was a significant positive correlation of alpha diversity (level of soil cores) and *C. scoparius* coverage (t = 2.289; P = 0.0221) as well as a significant correlation for alpha diversity and log-transformed distance from the extracted soil core to the base of the closest *C. scoparius* (t = -3.025; P = 0.0025) (Table 1; accompanying t values in Appendix B4). There was no significant correlation between the alpha diversity of Ascomycota and *C. scoparius* coverage, yet a highly significant correlation between the alpha diversity of all OTUs excluding Ascomycota and *C. scoparius* coverage (t = 4.662; P < 0.0001) (Appendix B5).

For average alpha diversity of all fungi at the plot-level, there was a significant increase over *C. scoparius* % coverage and an associated significant decrease over log-transformed distance from the extracted soil core to the closest mature *C. scoparius* (Figure 4). At the plot-level, average Ascomycota alpha diversity was not correlated with *C. scoparius* coverage, however all fungal OTUs excluding Ascomycota, and most notably Basidiomycota and Glomeromycotina, increased in alpha diversity over *C. scoparius* coverage (Figure 5). A positive correlation was likewise found

between *C. scoparius* coverage and Chytridiomycotina alpha diversity (R^2 = 0.41; P = 0.0040), as well as the alpha diversity of 'strict' saprotrophs (Figure 5).

The results were generally robust to the method of measuring *C. scoparius* cover and the definition of functional guilds. Average alpha diversity of all fungi at the plot-level had a similar response to mature *C. scoparius* (R^2 = 0.25; P = 0.0361) compared with immature *C. scoparius* (R^2 = 0.22; P = 0.0476) (Appendix B6). When substituting *C. scoparius* % coverage with the log-transformed distance from the extracted soil core to the closest mature *C. scoparius* (mm), there were no qualitative changes in any results related to alpha diversity (Appendix B7). Despite the overall increase in average alpha diversity which correlates with *C. scoparius* coverage, the plot with the highest gamma diversity (MW19) had no *C. scoparius* (Appendix B2).

Relative changes in average alpha (α) diversity at the level of plots

The ratio of mean Basidiomycota alpha diversity (per plot) compared to the mean alpha diversity of all fungi (per plot) increased over *C. scoparius* coverage (R^2 = 0.23; P = 0.0462). Similar relative increases in mean alpha diversity (per plot) over *C. scoparius* coverage could be observed for Glomeromycotina (R^2 = 0.50; P = 0.0010) and Chytridiomycotina (R^2 = 0.41; P = 0.0049) yet not for other fungal taxa nor for saprotrophs or symbiotrophs, although there was a non-significant increasing trend observed for 'strict' saprotrophs (R^2 = 0.20; P = 0.0660).

Overall beta (β) diversity

For all fungal OTUs, there was no significant change in beta diversity over *C. scoparius* coverage. When looking at individual fungal taxa and functional guilds, plots with higher *C. scoparius* coverage showed decreased Glomeromycotina and Basidiomycota beta diversity, whereas the beta diversity of Mucoromycotina increased with *C. scoparius* coverage (Figure 6). There was no qualitative change in results when substituting *C. scoparius* coverage with the log-transformed distance from the extracted soil core to the closest mature *C. scoparius* (Appendix B9). The beta diversity of Chytridiomycotina (R^2 = 0.32; P = 0.0180) and saprotrophs (R^2 = 0.26; P = 0.0297) both decreased in plots with higher *C. scoparius* coverage, although in both cases the method of measuring *C. scoparius* determined whether or not a significant change could be observed.

While 'strict' ectomycorrhizal fungi showed no change in beta diversity over *C. scoparius* coverage, an increase in beta diversity over *C. scoparius* coverage was found for 'loose' ectomycorrhizal fungi (i.e., ectomycorrhizal fungi which shared one or several different functional traits) (R^2 = 0.31; P = 0.0169).

Plant pathogens

Gamma. No change in gamma diversity was observed for 'strict' plant pathogens over *C. scoparius* coverage. When adapting the 'loose' definition of plant pathogens, the gamma diversity of 'loose' plant pathogens relative to the gamma diversity of all fungal OTUs decreased over *C. scoparius* coverage (R^2 = 0.35; P = 0.0100).

Alpha. There was a significant positive correlation of alpha diversity (level of soil cores) and *C. scoparius* coverage for 'strict' plant pathogens (t = 3.014; P = 0.0026) (Table 1). Increases in *C. scoparius* coverage correlated with increased mean alpha diversity of 'strict' plant pathogens (Figure 5). The ratio of mean 'strict' plant pathogen alpha diversity (per plot) compared to the mean alpha diversity of all fungi (per plot) increased over *C. scoparius* coverage (R^2 = 0.29; P = 0.0213).

Beta. Beta diversity of 'strict' plant pathogens decreased over *C. scoparius* coverage (R^2 = 0.50; P = 0.0010) (Figure 6) and a corresponding strong correlation was found between the beta diversity of 'strict' plant pathogens and the log-transformed distance to closest mature *C. scoparius* (R^2 = 0.53; P < 0.0001).

Proportional abundances

At the level of soil cores, the proportional abundance of Ascomycota (t = -2.871; P = 0.0041) and 'strict' symbiotrophs (t = -2.157; P = 0.0310) decreased with *C. scoparius* coverage while the proportional abundance of Mortierellomycotina (t = 2.638; P = 0.0083) and 'strict' plant pathogens (t = 2.767; P = 0.0057) increased with *C. scoparius* coverage (Table 1).

The increase in the proportional abundance of 'strict' plant pathogens could also be seen across plots (R^2 = 0.30; P = 0.0191) and although the proportional abundance of Glomeromycotina was unresponsive to *C. scoparius* at the level of soil cores, the proportional abundance of Glomeromycotina was greater in plots with higher *C. scoparius* coverage (R^2 = 0.26; P = 0.0299) (Figure 5).

For both Glomeromycotina and Mucoromycotina, there was an increase in the proportion of rarer OTUs (OTUs occurring in less than half of plots) over *C. scoparius* coverage per plot (Figure 7) and corresponding increases in the number of rarer OTUs over *C. scoparius* coverage (Appendix B10).

The effect of C. scoparius on community composition and OTU occupancy

Generally, plots with high *C. scoparius* coverage tended to form more homogeneous communities compared with more heterogeneous communities in plots with low to mid-range *C. scoparius* coverage (Figure 8).

The 20 most abundant OTUs across all three plots without *C. scoparius* coverage were exclusively Ascomycota or Basidiomycota, however Mortierellomycotina and Mucoromycotina OTUs were within the 10 most common OTUs from all three plots with highest *C. scoparius* coverage (Appendix B11). Nine out of 10 of the most dominant Ascomycota in the three plots without *C. scoparius* remained within the 20 most dominant Ascomycota in the three plots with most *C. scoparius*. Among the most abundant Mortierellomycotina OTUs (Appendix B12), 14 out of 20 were shared between the three plots with highest *C. scoparius* coverage and three plots without *C. scoparius*.

The most abundant Glomeromycotina OTU belonged to the family of Glomales according to Morton and Redecker (2001). The five most abundant Glomeromycotina OTUs across the three plots without any *C. scoparius* coverage were all *Glomeraceaea* sp. from the family of Glomeraceae. In contrast, only one of the five most abundant Glomeromycotina OTUs from the three plots with highest *C. scoparius* coverage was a *Glomeraceae* sp., the other four belonging to the families of Ambisporaceae, Archaeosporaceae and Claroideoglomeraceae.

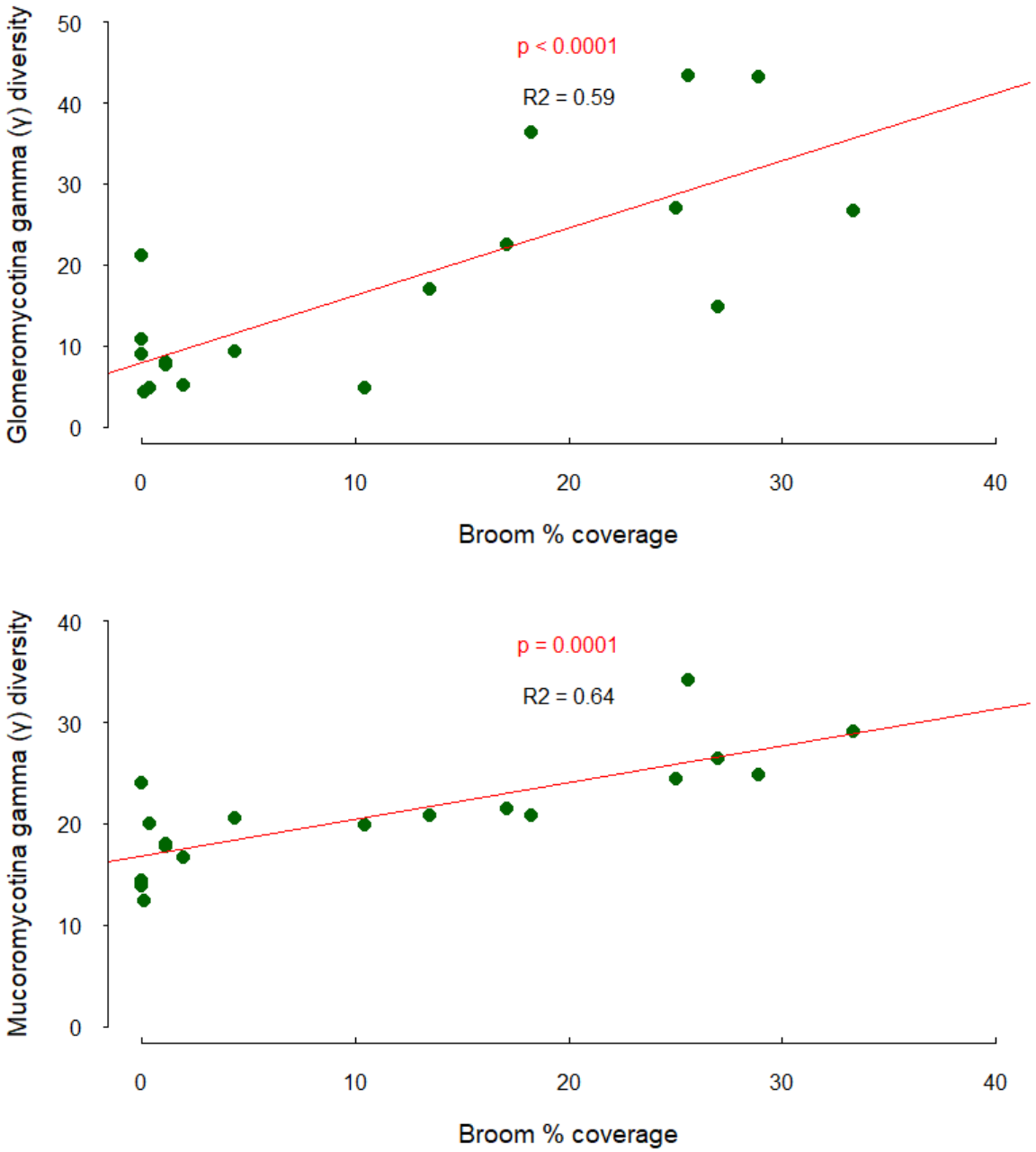


Figure 3. Gamma (plot-level, n = 18) diversity of Glomeromycotina (above) and Mucoromycotina (below) over *C. scoparius* % coverage. *P* and R^2 values are given in the plots. Corresponding plots for gamma diversity over log-transformed distance from the extracted soil to the closest mature *C. scoparius* (mm) are given in Appendix B3.

Table 1. Linear mixed-effect model results for alpha diversity (level of soil cores) and different measurements of *C. scoparius* density, i.e., *C. scoparius* % coverage and log-transformed distance between the extracted soil core and the stem of the closest *C. scoparius* (mm). *P* value estimates are likewise given for proportional abundance and different measurements of *C. scoparius* density, except for all fungi (indicated by "**.**"). Accompanying *t*-values are compiled in Appendix B4. ECM = Ectomycorrhizal fungi. Arbuscular mycorrhizal fungi (AMF) is not included in the table as OTUs for AMF according to FUNGuild (Nguyen *et al.* 2016) matched exactly with the OTUs for Glomeromycotina according to the UNITE public database (Nilsson *et al.* 2018).

	Alpha Diversity		Proportional Abundance	
	Broom % Coverage	log(Distance to Broom)	Broom % Coverage	log(Distance to Broom)
All fungi	**0.0221**	**0.0025**	**.**	**.**
Ascomycota	0.5570	**0.0196**	**0.0041**	0.4754
Basidiomycota	**< 0.0001**	**0.0013**	0.1227	0.2262
Glomeromycotina	0.1350	0.9485	0.1773	0.5797
Mortierellomycotina	**0.0006**	0.5088	**0.0083**	0.5281
Chytridiomycotina	**0.0001**	**0.0035**	0.1324	**0.0049**
Mucoromycotina	0.4903	0.5558	0.2077	0.7447
Antagonists	**0.0023**	0.0573	0.3491	0.4306
Symbiotrophs	0.9183	0.0631	**0.0310**	0.5817
Saprotrophs	**0.0464**	**0.0042**	0.3360	0.6416
Plant pathogens	**0.0026**	0.0870	**0.0057**	**0.0020**
Pathogens of fungi	0.3686	0.4951	0.0908	0.7617
ECM (FUNGuild)	0.7239	0.1358	0.4264	0.9678

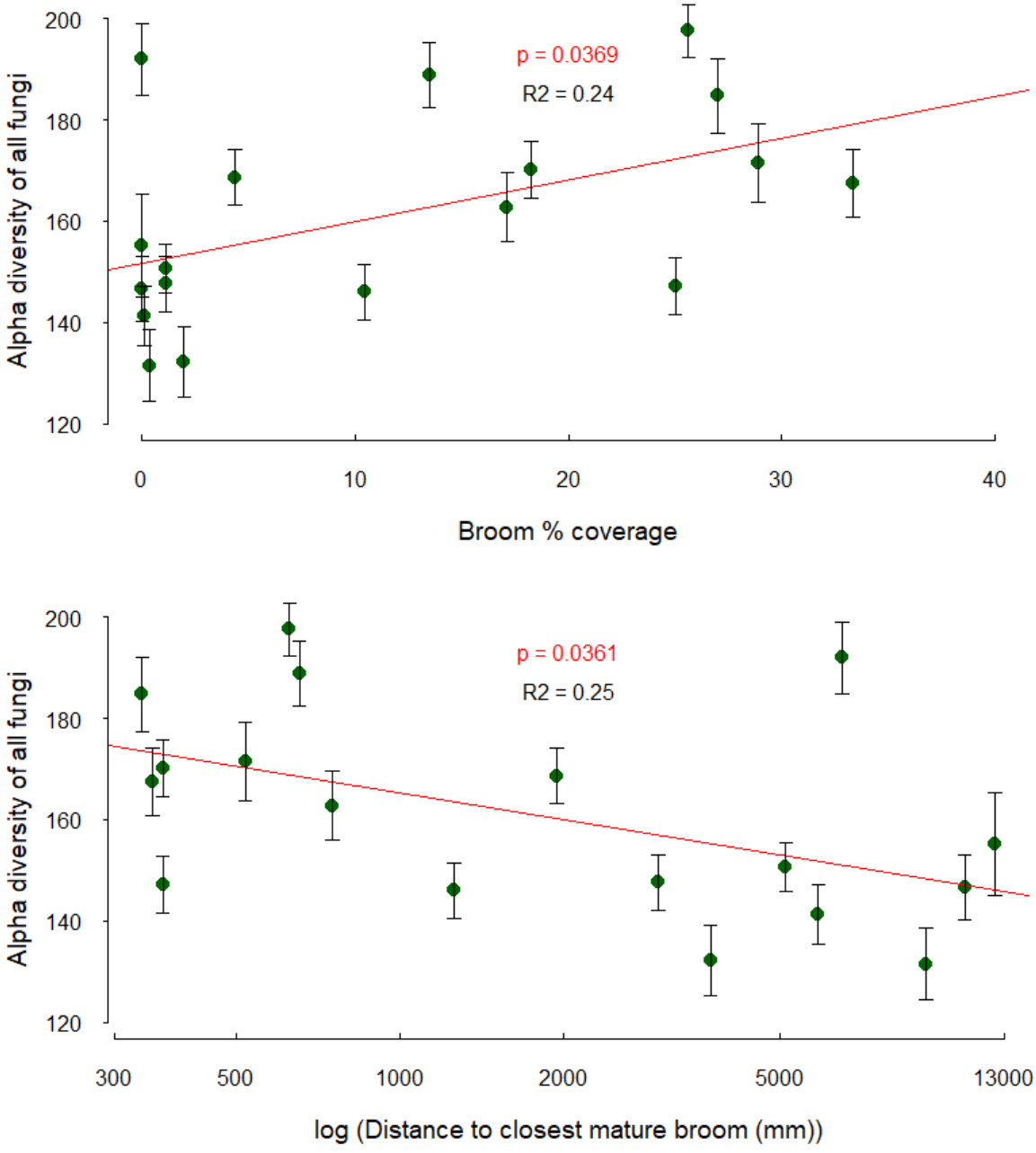
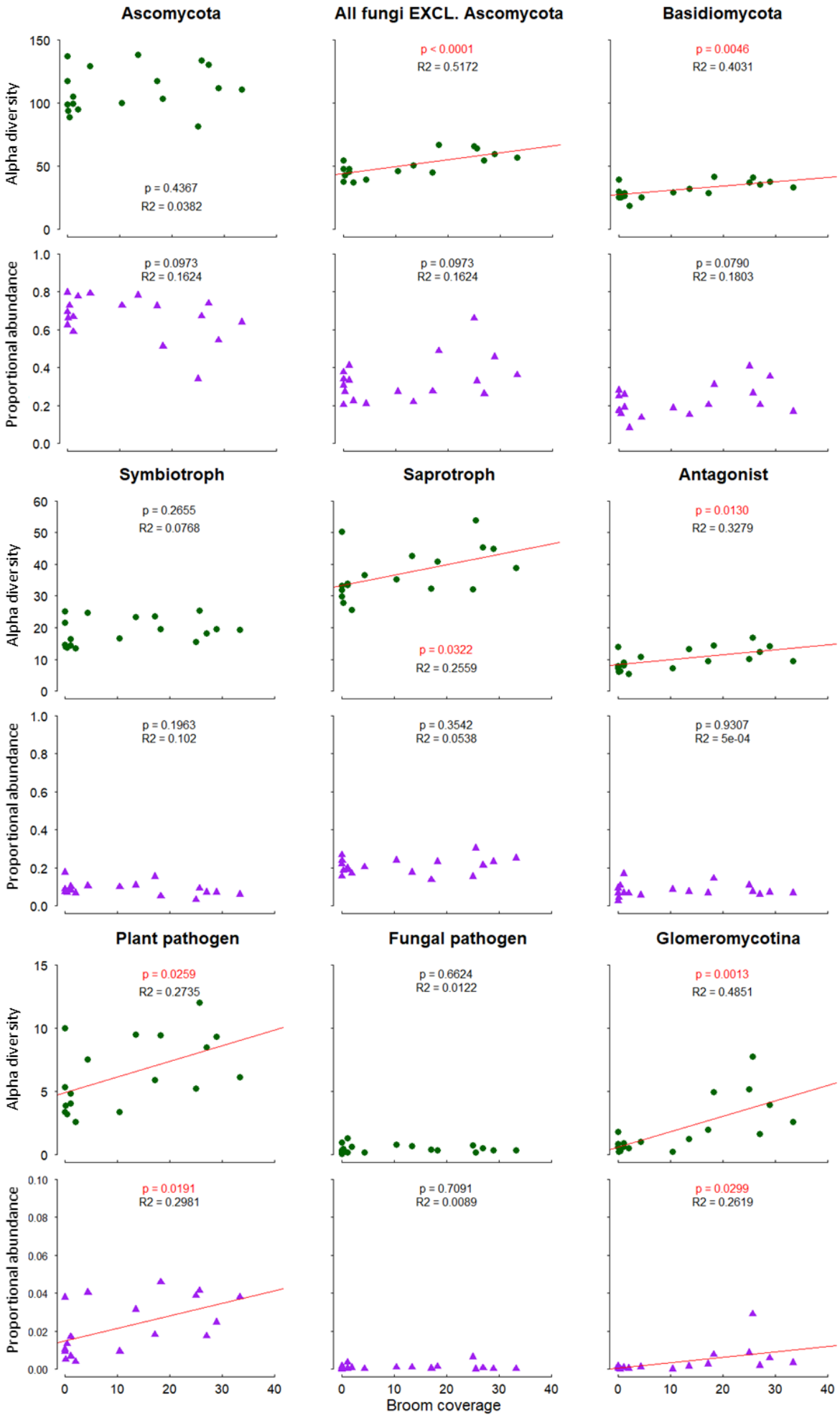


Figure 4. Average alpha diversity ± SE (min. 23 soil cores per plot) of all fungal OTUs over *C. scoparius* % coverage (above) and average alpha diversity of all fungal OTUs over the distance from the extracted soil core to the closest mature *C. scoparius* (below). *P* and R^2 values are given in the plots. A plot for average alpha diversity per plot over log-transformed distance of closest *C. scoparius* (whether mature or immature) is given in Appendix B6.

Figure 5. *[Next page]* Average alpha diversity and proportional abundance of fungal OTUs (per plot) according to fungal taxa and functional traits over *C. scoparius* % coverage. Regression lines are shown when $P < 0.05$ and *P* and R^2 values are given in the plots. All OTUs were 'strictly' classified into functional guilds (results with 'loose' classifications, i.e., with overlap, are presented in Appendix B8).


Ascomycota
All fungi EXCL. Ascomycota
Basidiomycota
Alpha diversity
p = 0.4367
R2 = 0.0382
p < 0.0001
R2 = 0.5172
p = 0.0046
R2 = 0.4031
Proportional abundance
p = 0.0973
R2 = 0.1624
p = 0.0973
R2 = 0.1624
p = 0.0790
R2 = 0.1803
Symbiotroph
Saprotroph
Antagonist
p = 0.2655
R2 = 0.0768
p = 0.0322
R2 = 0.2559
p = 0.0130
R2 = 0.3279
p = 0.1963
R2 = 0.102
p = 0.3542
R2 = 0.0538
p = 0.9307
R2 = 5e-04
Plant pathogen
Fungal pathogen
Glomeromycotina
p = 0.0259
R2 = 0.2735
p = 0.6624
R2 = 0.0122
p = 0.0013
R2 = 0.4851
p = 0.0191
R2 = 0.2981
p = 0.7091
R2 = 0.0089
p = 0.0299
R2 = 0.2619
Broom coverage

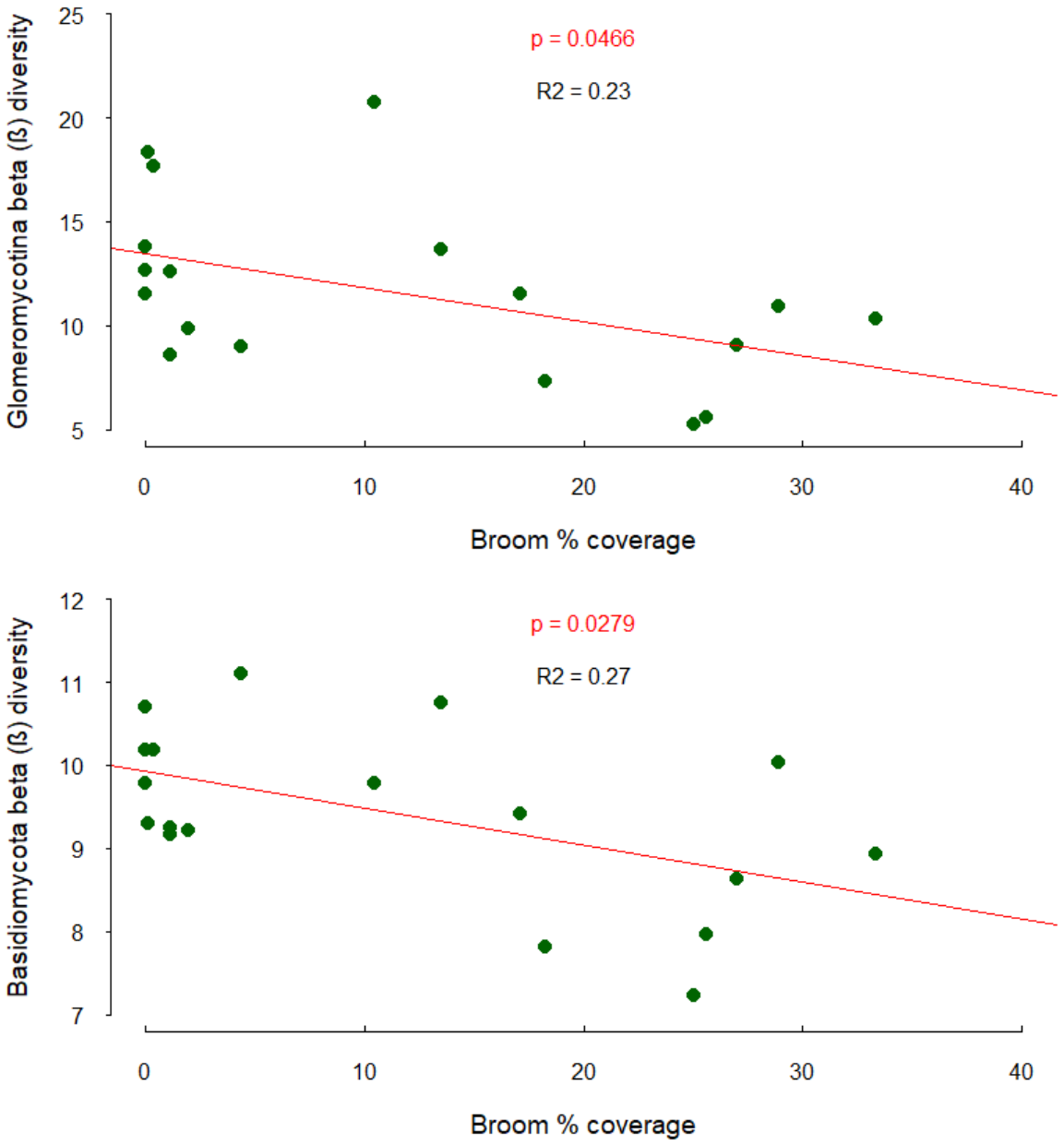


Figure 6. Beta (β) diversity of Glomeromycotina and Basidiomycota over *C. scoparius* % coverage (below) and Mucoromycotina and plant pathogens over *C. scoparius* % coverage (next page). *P* and R^2 values are given in the plots.

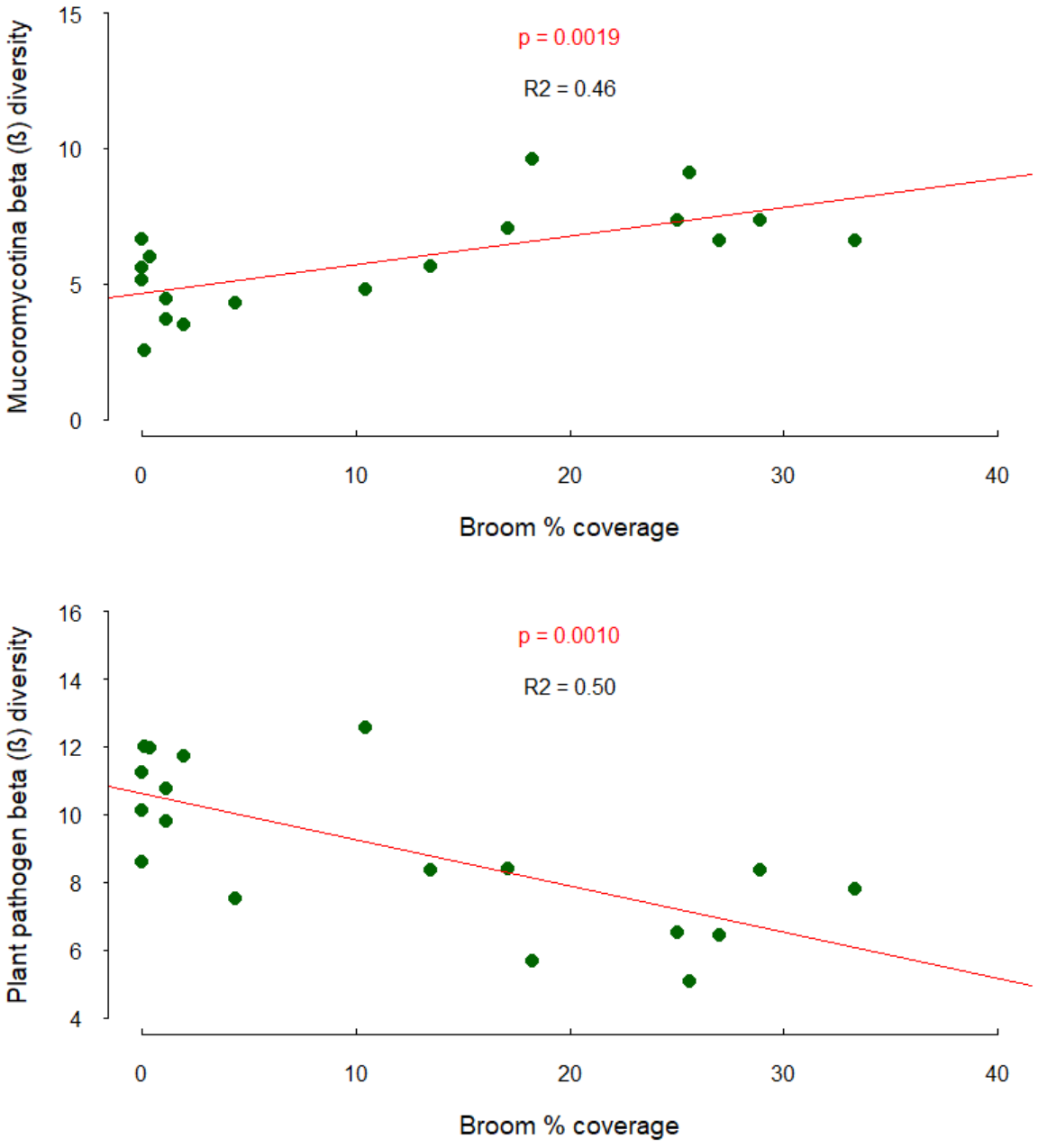


Figure 6. *[Continued]*

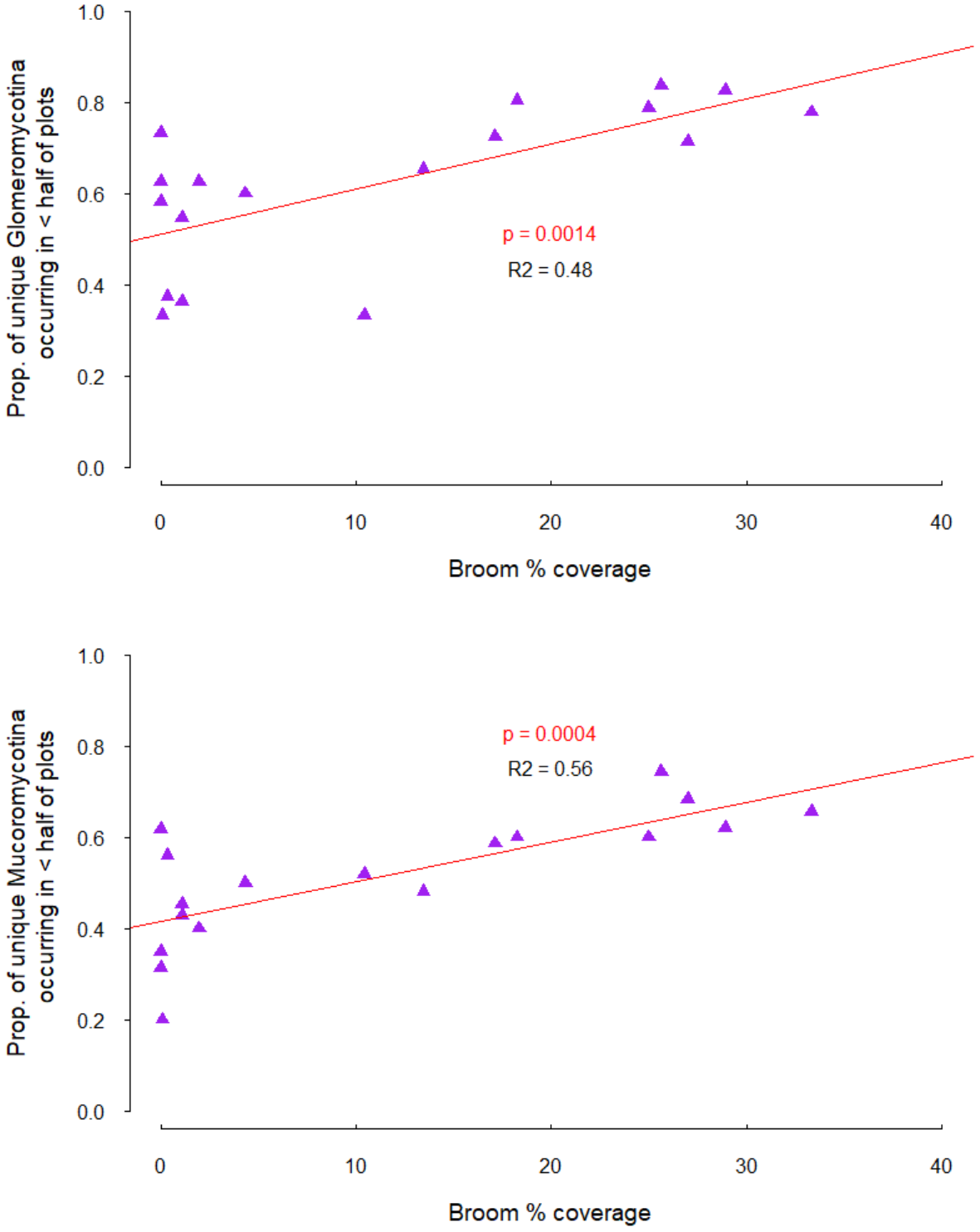


Figure 7. Proportion of unique Glomeromycotina and Mucoromycotina OTUs occuring in less than half of all plots (relative to all Glomeromycotina and Mucoromycotina OTUs per plot) over *C. scoparius* % coverage. *P* and R^2 values are given in the graphs.

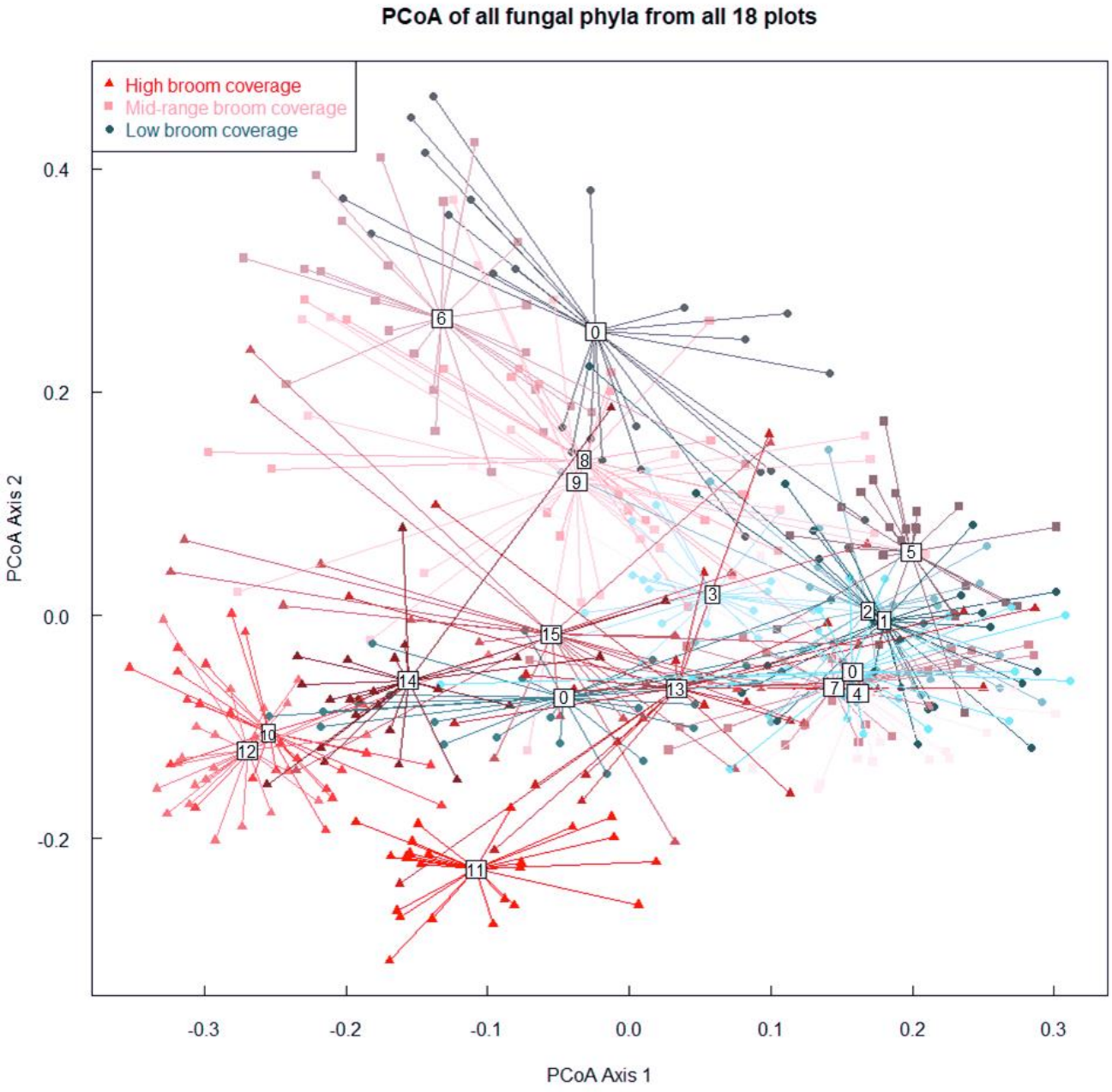


Figure 8. Principle Coordinates Analysis (PCoA) of all soil cores (n = 431) across all plots (n = 18) coloured according to *C. scoparius* coverage. The community matrix for all fungal OTUs was Wisconsin-transformed prior to scaling. Rank order of the plots from lowest to highest *C. scoparius* cover is given at the centroid of each plot (with three plots without *C. scoparius* labelled "0").

Discussion

In contrast with my hypotheses and the widespread view that biological invasions are associated with a loss of diversity, my results show that *C. scoparius* invasion increased fungal diversity at the average point-scale per plot (average alpha diversity), including increasing the diversity of plant pathogens.

Results differed according to at which scale the study was undertaken (Table 2) and most notably for Glomeromycotina which showed no response to *C. scoparius* at the level of soil cores yet increased with *C. scoparius* cover in all of the diversity metrics at the plot scale (average alpha diversity and beta and gamma diversity) as well as in proportional abundance and in number of rare AMF OTUs. These differences related to the scale at which the study was undertaken may indicate that relatively large increases in *C. scoparius* biomass are required before any noticeable effect on fungal diversity may be observed.

Response of fungal gamma and alpha diversity to C. scoparius

A possible reason why increases in both gamma and alpha diversity were observed across several fungal taxa (Table 2) could be that *C. scoparius* enriches the productivity of the soil environment (i.e., increases biomass generation), which can in part be supported by the observed increase in average saprotroph alpha diversity per plot. Although community productivity generally increases with the number of species in local communities (Balvanera *et al.* 2006, Maron *et al.* 2011), plots with high *C. scoparius* cover likely have a greater proportion of topsoil which has undergone increased C and N input, to the extent that soil beneath *C. scoparius* has a higher productivity than uninvaded grassland. Although I can only infer a loss in plant diversity caused by increased *C. scoparius* coverage, highly productive alien plant species have been known to simultaneously increase productivity even while reducing local plant species diversity (Vilà *et al.* 2011).

The diversity of AMF has long been a key indicator of soil productivity (Van Der Heijden *et al.* 1998) and AMF have an important role in increasing the competitive ability of certain invasive plants (Zhang *et al.* 2018). Based on the correlation of AMF diversity and invasive plant success (Zhang *et al.* 2018), it is probable that the elevated AMF diversity following *C. scoparius* invasion could be one of the factors enabling *C. scoparius'* spread. This increased diversity of AMF correlated with results in Bahram *et al.* (2020), who likewise found that sites with higher AMF diversity harboured more saprotrophs (and plant pathogens) in the topsoil. Finding more soil saprotrophs (i.e., decomposers) surrounding *C. scoparius* is broadly in line with higher decomposition rates found in ecosystems with higher AMF dominance (Tedersoo and Bahram 2019). My results however differ from those in Bahram *et al.* (2020), who found lower fungal diversity in plant

monocultures, as plots in my study which had near-monocultures of *C. scoparius* showed increases in both gamma and average alpha diversity across several fungal taxa (Table 2).

Response of fungal beta diversity to C. scoparius

Whereas *C. scoparius* coverage correlated with an overall increase in alpha diversity, a general decrease in beta diversity over *C. scoparius* coverage was observed (Table 2). This decrease in beta diversity may suggest that *C. scoparius* simultaneously increased local fungal diversity but also homogenised the fungal community, resulting in more species in each soil core yet less variability across cores. Aboveground homogeneity has been linked to decreased fungal beta diversity (Zak and Willig 2004, Bachelot *et al.* 2016) and there has generally been a correlation between the biodiversity of groups of directly or indirectly interacting organisms (Gaston 2000, Scherber *et al.* 2010, Peng *et al.* 2019). As such, the decrease in beta diversity is probably more related to *C. scoparius'* aboveground dominance (i.e., increased plant homogeneity), rather than to an increase in soil productivity induced by *C. scoparius*.

Response of plant pathogens to C. scoparius

Pathogens of fungi did not significantly respond to *C. scoparius* coverage, however increased *C. scoparius* coverage correlated with an increase in the alpha diversity of plant pathogens (both within individual soil cores and across plots) and a decrease in plant pathogen beta diversity (Table 2).

The diversity and composition of plant pathogens is tightly connected to plant communities (Mangelsdorff *et al.* 2012, Hantsch *et al.* 2013, Latz *et al.* 2016). Plant pathogen alpha diversity has been known to be influenced by plant richness, as a higher richness of host plants (i.e., a broader niche) is likely accompanied by a higher richness of plant pathogens (Bond and Chase 2002). It has therefore been proposed that a reduction in plant diversity will reduce the diversity of plant pathogens (Gossner *et al.* 2016), particularly in AMF dominated habitats which typically experience greater antagonism from their associated soil microbiota compared with ectomycorrhizal dominated habitats (Teste *et al.* 2017, Kadowaki *et al.* 2018). As *C. scoparius* formed near-monocultures in some plots, finding greater plant pathogen alpha diversity with increased *C. scoparius* coverage was again unexpected.

Plant pathogen alpha diversity is frequently higher in host plants with a history of agricultural use or host plants with wide geographical ranges (Mitchell *et al.* 2010, Kamiya *et al.* 2014). Plant pathogen alpha diversity has also been known to accumulate according to how long an exotic plant has been established in New Zealand (Diez *et al.* 2010), partly as while plants become more widespread, the plants will also have an increased probability of encountering more pathogens (Hawkes 2007). *Cytisus scoparius* does have a broad geographic range in New Zealand (Syrett *et al.* 1999), where *C. scoparius* was naturalized by 1872 (Owen 1998). It follows that both the geographic

range of *C. scoparius* and the shrub's long history as an invasive plant in New Zealand could have both led to gradual accumulations in plant pathogens to the extent that the richness of *C. scoparius'* pathogens supersedes the pathogen richness of uninvaded grasslands, despite the uninvaded grasslands having a higher richness of host plants (Bond and Chase 2002). This process could however work both ways to the effect that *C. scoparius* encounters both "friends and foes" throughout its spread. AMF associating with an invasive plant has in some cases been shown to improve plant growth and resistance (Zhang *et al.* 2018, Chen *et al.* 2019a) (although see Reinhart *et al.* (2017) concerning limitations). *Cytisus scoparius* could have accumulated different species of fungi (particularly Glomeromycotina), which may counteract antagonistic effects and thereby enable increases in multiple functional groups of fungi.

It is however unlikely that *C. scoparius'* large-scale accumulation of pathogens (and potentially mutualists) over time is the sole reason as to why an increase in pathogen diversity was observed. High connectivity of invasive *C. scoparius* populations would also enable the spread of pathogens. A plant's life strategy and physical size (Van der Putten *et al.* 1993, García-Guzmán and Heil 2014) as well as N-fixation are factors which could have influenced the studies' outcome. Shading by *C. scoparius* can be expected to create moister and more moderated conditions which would likely aid in water retention (Danner and Knapp 2003). As soil water availability is considered one of the strongest predictors of fungal richness at a global scale (Tedersoo *et al.* 2014) and as soil water retention increases with organic matter (Gupta and Larson 1979, Emerson 1995) (although see Rawls *et al.* (2003) concerning limitations), a likely increase in soil organic matter caused by *C. scoparius* could be accompanied by increases in fungal diversity, including plant pathogen diversity.

Response of fungal proportional abundance to C. scoparius

For two rare fungal taxa (Glomeromycotina and Mucoromycotina), there was an increase in the proportion of rarer OTUs over *C. scoparius* coverage, which was also reflected in an increase in gamma diversity. It was still unexpected that soil under *C. scoparius* should harbour more unique OTUs compared to surrounding soil with higher plant diversity, although finding a higher proportion of novel fungal OTUs in soil affected by an invasive plant (compared to uninvaded soil) has been documented (Anthony *et al.* 2017).

Experimental design considerations

Although it was my aim to sample across a natural density gradient of an existing *C. scoparius* invasion rather than to study the effect of artificially created plant monocultures on soil biodiversity (e.g., Gornish *et al.* (2016), Gibbons *et al.* (2017)), a major downside of not using experimental monocultures is that I cannot be certain whether *C. scoparius* caused the observed changes in soil fungal communities or merely responded to existing soil fungal communities. *Cytisus scoparius* in

my field-site did however commonly occur in dense irregular patches, often near grassland uninvaded by *C. scoparius*. The natural distribution of *C. scoparius* was generally indicative of patchy seed dispersal followed by spread from dense points, which may suggest that the expansion of *C. scoparius* was not reliant on existing soil communities.

Microbial communities are known to differ between plant species (Innes *et al.* 2004), and even between genotypes within species (Kowalchuk *et al.* 2006). Although I sampled a typical grassland site in which *C. scoparius* occurs commonly across New Zealand (Bellingham and Coomes 2003), I cannot claim that *C. scoparius* invasion in different sites will follow the same pattern.

Metabarcoded eDNA data is known to be semi-quantitative (Martínez-García *et al.* 2015) as OTU frequency does correlate to a certain extent with species relative abundance (Taberlet *et al.* 2018). Although the diversity and load of plant pathogens can be closely linked (Hantsch *et al.* 2014), plant pathogen OTU abundance should still not be strictly translated to pathogen load on plants (Torchin and Mitchell 2004). As measurements for total fungal biomass are not included within this study, I cannot interpret increases in the proportional abundance of plant pathogens and Glomeromycotina over *C. scoparius* coverage as increases in the total biomass of plant pathogens and Glomeromycotina, and consequently place more importance on diversity estimates.

There are many different methodological variations that might have influenced results, including the size and depth of soil cores, the decision not to remove relic DNA (Carini *et al.* 2020) and the number of PCR replicates undertaken for each soil extract (Dopheide *et al.* 2019). My next chapter on eDNA pooling will further explore how the handling of soil extracts pre-PCR impacts observed outcomes.

Of all New Zealand fungi known to associate with *C. scoparius*, 65.1% (according to nzfungi2.landcareresearch.co.nz) had corresponding entries in FUNGuild, implying that FUNGuild likely underestimated the number of fungal OTUs. Another limitation of existing fungal databases is that fungal species known only from sequence data are not handled well (Nilsson *et al.* 2019b), nor necessarily inform whether fungi (and particularly cryptic species of fungi) are native to New Zealand or not. It is therefore difficult to study whether OTUs found in the proximity of *C. scoparius* are introduced or native (however, see Bogar *et al.* (2015) concerning a possible way to assess fungal geographic origins). Although I would require an analysis of species origin for confirmation, an increase in the proportion of unique OTUs found in *C. scoparius* yet not in surrounding uninvaded grassland could possibly be an indicator of *C. scoparius* co-invading with belowground mutualists, pathogens and commensals (Nuñez and Dickie 2014).

Conclusions and applications

For plant pathogens, Basidiomycota and Chytridiomycotina, having no increase with *C. scoparius* coverage in gamma diversity alongside an increase in average alpha diversity per plot while beta diversity decreased with *C. scoparius* coverage could possibly be regarded as an indicator of coalescence between previously separated fungal communities (Rillig *et al.* 2015). Rather than one existing fungal community outcompeting the other (Foster and Bell 2012), a new community is formed around *C. scoparius* composed of fungi spreading alongside *C. scoparius* and the existing community in uninvaded grassland.

It is possible that invasive plants accumulate soil pathogens which inhibit native plants (Mangla *et al.* 2008). Knowing that *C. scoparius* increases the diversity of putative plant pathogens across plots was an initial surprise given that most plants in my previous soil legacy experiment (Chapter 2), including natives, benefited from being planted in soil with *C. scoparius'* legacy, yet it has been observed that the diversity of putative soil pathogens is not necessarily indicative of plant growth (Hawkes 2007, Van der Putten *et al.* 2013).

My results highlight that studying fungal communities at different scales might lead to dissimilar outcomes and puts a spotlight on the need to have a robust experimental design prior to commencing an eDNA survey (Dickie *et al.* 2018, Zinger *et al.* 2019). My next chapter will delve deeper into the methodological processes underlying eDNA surveys.

Table 2. Summary of the effect of *C. scoparius* coverage on fungal diversity. Effect of *C. scoparius* at the scale of soil cores is measured via linear mixed-effect model estimates (with plot as a random variable). Effect of *C. scoparius* on the regional scale is measured across all 18 plots. '—' indicates no observed correlation (i.e., $P > 0.05$). All fungal functional traits follow 'strict' classifications according to FUNGuild (Nguyen *et al.* 2016). When the 'strict' classification differs from the 'loose' classification, the response of the 'loosely' grouped functional guild is shown within parentheses. Relative richness was calculated by dividing the plot-level mean alpha diversity of a group of interest (e.g., Basidiomycota) by the plot-level mean alpha diversity of all fungi. ECM = Ectomycorrhizal fungi.

	Scale of sampling plots (n = 18)					Scale of soil cores (n = 431)	
	Mean alpha (α) diversity	Beta (β) diversity	Gamma (γ) diversity	Proportional abundance	Relative richness	Alpha (α) diversity	Proportional abundance
All fungi	↑	–	–	NA	NA	↑	NA
All fungi EXCL. Ascomycota	↑	–	↑	–	–	↑	↑
Ascomycota	–	–	–	–	–	–	↓
Basidiomycota	↑	↓	–	–	↑	↑	–
Glomeromycotina	↑	↓	↑	↑	↑	–	–
Mortierellomycotina	–	–	–	–	–	↑	↑
Chytridiomycotina	↑	↓	–	↑	↑	↑	–
Mucoromycotina	–	↑	↑	–	–	–	–
Antagonists (general)	↑	↓	–	–	↑	↑	– (↑)
Plant pathogens	↑	↓	–	↑	↑	↑	↑
Pathogens of fungi	–	– (↑)	–	– (↓)	–	–	–
Symbiotrophs	–	–	–	–	–	– (↑)	↓
Saprotrophs	↑	–	–	–	–	↑	–
ECM	–	– (↑)	–	–	–	–	– (↓)

Chapter 4: Consequences of environmental DNA pooling

Abstract

DNA-based techniques are increasingly used to assess biodiversity both above- and belowground. Most effort has focussed on bioinformatics and sample collection, whereas less is known about the consequences of mixing collected environmental DNA (eDNA), post-extraction and pre-PCR. We applied varying degrees of pooling to stand-alone eDNA samples collected across a non-native plant invasion density gradient, and compared the fungal communities of pooled and unpooled samples. Pooling soil eDNA decreased observable fungal rarefied richness in our samples, led to phylum-specific shifts in proportional abundance, and increased the sensitivity of detection for the invasive plant's overall impact on fungal diversity. We demonstrate that pooling fungal eDNA could change the outcome of similar eDNA studies where the aim is to: 1) identify the rare biosphere within a soil community, 2) estimate species richness and proportional abundance, or 3) assess the impact of an invasive plant on soil fungi. Sample pooling might be appropriate when determining larger-scale overarching responses of soil communities, as pooling increased the sensitivity of measurable effects of an invasive plant on soil fungal diversity.

Keywords

Diversity, environmental DNA, experimental design, fungal communities, metabarcoding, sampling, soil DNA extraction

Introduction

High throughput DNA sequencing technology (Caporaso *et al.* 2012) is increasingly used for determining the composition of ecological communities, both terrestrial and aquatic, and for testing ecological hypotheses (Holdaway *et al.* 2017). These approaches have the potential to revolutionize biodiversity and conservation monitoring (Lindahl *et al.* 2013). One technique in particular, DNA metabarcoding, can identify the presence of a multitude of species across a wide taxonomic range (Taberlet *et al.* 2012), which previously could only be achieved through the time-consuming morphological identification of individual organisms (Lawton *et al.* 1998). The growing use of DNA metabarcoding to sequence environmental DNA (eDNA, i.e., DNA extracted from soil, water, air or other substances) has brought to attention the need for in-field collection and sampling protocols as well as instructions on how to process obtained samples in the laboratory environment. There have been previous reviews of metabarcoding methods which focus on statistical replication in sampling (Lennon 2011), the processing of collected samples (Lear *et al.* 2018), as well as data reporting and bioinformatics analysis (Hiraoka *et al.* 2016). However, there are very few DNA metabarcoding studies on the effect of mixing extracted eDNA samples together, i.e., "pooling" samples prior to being sequenced. The most thorough study to date on determining the effect of eDNA pooling is possibly Avis *et al.* (2010), who mixed up to 20 pre-selected fungal species prior to molecular analysis. Given how common pooling is when undertaking community studies (Dickie *et al.* 2018) and given that sampling and subsequent pooling techniques are the basis on which valid inferences are dependent (Crawley 2015), the consequences of pooling large numbers of eDNA samples prior to sequencing deserves more attention.

Sample pooling is typically performed as a means of estimating the dominant species in a given area (Ellingsøe and Johnsen 2002), or in order to gather more information on the complexity of sampled biodiversity. In either case, pooling is closely associated with a loss of spatial variability information (Dickie *et al.* 2018), which can be of minor or major concern dependent on the research question. Two seemingly unavoidable downsides of pooling are a reduced ability to detect rare species, particularly for fungi in comparison to bacteria (Manter *et al.* 2010), as well as a reduced ability to estimate species richness (Kang and Mills 2006). A methodological issue surrounding the detection of rare species occurs at the PCR amplification stage of eDNA studies. As PCR is a competitive process (Siebert and Larrick 1992) where the level of amplification achieved is positively correlated with the amount of starting template DNA, species with relatively low abundance will undergo PCR amplification to a lesser degree than species with a relatively high

abundance. Pooling is likely to dilute rare DNA templates to the extent that amplification of rare templates may be insufficient for detection.

There are studies which suggest that pooling pre-PCR has little effect on the perceived community (Manter *et al.* 2010, Osborne *et al.* 2011) making sample pooling economically advantageous, yet it has also been observed that pooling decreases detected variability when compared to not pooling (Osborne *et al.* 2011). When presented with a pooled sample, it is possible to lessen the issue of decreased detected variability by amplifying several diluted subsets of the pooled sample (which would increase the likelihood that less abundant species are successfully amplified and detected), yet in such cases it might have been better to have instead taken multiple stand-alone samples allowing for additional spatial variability analyses (Dickie *et al.* 2018). This is because a rare species in a given area can be 1) found in low abundance but ubiquitously distributed, or 2) found in high abundance at fine scales but heterogeneously distributed (Green *et al.* 2004). Such spatial distributions could be characteristic to different fungal taxa, for instance, it has been observed that wood-inhabiting members of the fungal phylum Ascomycota are more specific to certain tree species compared with the more homogeneously distributed fungal phylum Basidiomycota (Purahong *et al.* 2018).

Among studies which have directly explored the consequences of pooling eDNA samples (Ellingsøe and Johnsen 2002, Manter *et al.* 2010, Osborne *et al.* 2011, Song *et al.* 2015, Sato *et al.* 2017), only Manter *et al.* (2010) and Song *et al.* (2015) deal with soil-extracted fungal samples. Song *et al.* (2015) used two pooled samples in their experiment, each created by mixing four soil samples before eDNA extraction; they observed that although pooled samples had a higher fungal OTU richness compared with stand-alone samples, computational pools created by combining stand-alone samples showed higher OTU richness compared with the physical pool. In the case of Manter *et al.* (2010), a community fingerprinting (ribosomal intergenic spacer analysis) approach was adopted using soil from three very dissimilar sampling sites from both hemispheres; they found that in their sample sets, fungi were typified by locally abundant but spatially rare phylotypes, whereas bacteria in their study were typified by locally rare but spatially ubiquitous phylotypes. As a result, pooling would differentially influence their plot comparisons and would mask a significant proportion of their detectable microbial community, particularly for fungi due to their higher spatial heterogeneity. Sato *et al.* (2017) tested whether pooling of eDNA samples from four Japanese lakes could be used to evaluate the biodiversity of freshwater fishes and found that their pooling strategy was unsuitable for estimating species richness, yet had potential for among-site comparison of their fish communities. Osborne *et al.* (2011) studied how their pooling strategies influenced the detected composition of bacterial communities in three distinct Australian land-use plots, finding that as few as 8 or 10 cores per plot was sufficient to detect significant differences between the bacterial communities from their three study sites.

In a study on soil bacterial communities dug up from a Danish forest and identified via a denaturing gradient gel electrophoresis approach (DGGE), Ellingsøe and Johnsen (2002) observed that using larger soil samples (which to a certain degree are comparable with pooled samples) could be more appropriate when studying anthropogenic activities on bacterial community structure when compared to smaller soil samples where chance variations could play a larger role. Soil resources (e.g., organic matter) and soil properties can often vary at a smaller scale than sample volume (Cappai *et al.* 2017, Evgrafova *et al.* 2018) and could be considered a source of unexplained variance which may conceal the larger-scale effect of environmental drivers (e.g., anthropogenic effects, invasive species impacts). In relation to fungi, the relative abundance of the fungal phylum Ascomycota has been shown to be negatively correlated with soil organic C in agricultural fields, while the relative abundance of the fungal phyla Basidiomycota is positively correlated with soil organic C (Zebarth *et al.* 2018). In this example, pooling samples could have a 'double-edged sword' effect in terms of studying the soil community: on the downside, sample pooling could dampen the smaller-scale effect of soil organic C and distort measurements of relative abundance, yet on the upside enhance the detection of an overarching effect such as anthropogenic activity.

Although some valuable insights on soil eDNA pooling have been provided via a small number of mixed samples pooled pre-extraction (Song *et al.* 2015), to the best of my knowledge, there is yet no comprehensive study which examines how sample pooling post-extraction affects the species richness and proportional abundance measurements of fungal eDNA, nor how identifying the presence of an ecological gradient (e.g., the effect on an encroaching invasive plant species on belowground species richness) might be hindered or exaggerated by pooling eDNA samples. Given the cost of field sampling, wet-lab processing and sequencing, it is desirable to neither under- nor oversample when conducting an eDNA-based ecological survey. Methodologically sound eDNA sample preparation is the foundation for subsequent analyses and examining the consequences of sample pooling would be one way to help assess how reliable and reproducible an eDNA survey is. In this study, I applied four degrees of eDNA pooling to individual soil core extracts collected from six plots along an exotic plant's invasion gradient, followed by Illumina sequencing of indexed PCRs targeting the ribosomal internal transcribed spacer (ITS) region (Schoch *et al.* 2012) within soil fungi.

My aim was to investigate how varying degrees of eDNA sample pooling affects species richness and proportional abundance of soil organisms, specifically fungi, and to examine whether sample pooling enhances or dampens the overarching effect of an exotic plant (*C. scoparius*) on soil fungal communities. Considering the large variability of data curation steps found in eDNA analyses (Dickie *et al.* 2018, Calderón-Sanou *et al.* 2019), species richness estimations have been known to be sensitive to inaccuracies (Flynn *et al.* 2015, Dopheide *et al.* 2019), yet in contrast, studies which focus on comparing communities' composition are considered less prone to errors (Leray and

Knowlton 2015, Taberlet *et al.* 2018). I therefore chose to focus on how pooling affects proportional abundances within my sampled communities, particularly at the level of fungal phylum, as this observation would likely be more relatable to other fungal eDNA studies compared with species richness.

As a broader area would have been covered, it is reasonable to expect a pooled eDNA sample to have a higher species richness than any stand-alone sample that the pooled sample is composed of, however, I surmised this to be at the cost of a reduced detectability of the rare biosphere.

I hypothesised that:

- Although pooling eDNA samples is expected to increase species richness, a proportion of the "true" species richness is likely to become undetectable by DNA metabarcoding techniques because of pooling, partly due to over-dilution of rare DNA templates.

- Given that different fungal phyla are known to have broader or more restricted distributions (Purahong *et al.* 2018, Zebarth *et al.* 2018), sample pooling will cause distortions in the "true" proportional abundance of fungal taxa.

- As sample pooling will likely decrease within-plot variation of fungal communities, caused by multiple unaccounted abiotic and biotic factors, the use of pooled samples should therefore be more sensitive to larger scale between-plot comparisons, which in this case is the presence of an exotic plant (*Cytisus scoparius*) across an invasion gradient.

To test these hypotheses, I apply varying degrees of pooling to stand-alone eDNA samples systematically collected across an exotic plant's invasion gradient and compare the fungal communities of computationally pooled samples with my physically pooled samples (pooled after DNA extraction).

Methods

Study site and field experiment

The study site was located in the Saint James conservation area in New Zealand's South Island (-42.460273 Lat., 172.830938 Long.; elevation = 800–900 m.a.s.l.; mean annual temperature = 10.3°C; mean annual rainfall = 1158 mm, Hanmer forest weather station). *Cytisus scoparius* (Scotch broom) is widely spread throughout this region and a description of the site's vegetation is given in Broadbent *et al.* (2017). Permanent 20 × 20 m vegetation plots were laid out at the site by Manaaki Whenua – Landcare Research, following standard field protocols (Hurst and Allen 1993). For this experiment, I selected six permanent vegetation plots across a *C. scoparius* density gradient (i.e., two plots with low *C. scoparius* coverage, two plots with intermediate *C. scoparius* coverage and two plots with high *C. scoparius* coverage) and all plots were located within 1 km of each other. Field sampling took place from 14 February 2017 to 17 April 2017. For each of the six permanent vegetation plots, 24 individual georeferenced soil cores were taken, totalling in 144 spatially explicit soil samples. The six plots were a subset of those used in my natural survey of *C. scoparius* invasion (Chapter 3) and subsequent methods regarding how I undertook in-field soil sampling as well as measured *C. scoparius* coverage are identical to the methods in Chapter 3.

Figure 1 gives an overview of how each soil sample was processed, once brought back from the field site. Each ~250 g soil sample (stored at 4°C) was broken up manually and spread out evenly on clean paper. Using bleached forceps and spatulas, a ~10 g mixed soil sample was obtained by systematically extracting 10 × ~1 g subsamples from across the initial sample. The mixed subsampled soil did not contain any roots more than 5 mm in width or stones larger than 5 mm in diameter and obvious insects (e.g., ants, larvae) were avoided. The processed soils were kept frozen at -18°C until DNA extraction.

Experimental design and wet-lab processing

Both the kit used for soil DNA extraction and the chosen fungal primers were recommended by Lear *et al.* (2018). DNA extraction was performed on the 144 soil cores using DNeasy PowerSoil® HTP 96 Kits (Quiagen), according to the manufacturer's instructions and loading the maximum amount of recommended soil for DNA extraction (250 mg). As part of the PowerSoil® protocol, mechanical lysis of the soil samples was performed using a Spex® Sample Prep 1600 MiniG. Five µL subsamples of the 144 stand-alone soil extracts (at full concentration) were mixed in equal proportions to create pooled soil samples, composed of 3, 6, 12 or 24 combined soil extracts. I chose the pooling partitions in such a way that smaller partitions "fitted" into larger ones, creating "pools within pools" to enable more practical comparisons. Figure 2 shows the partitions based

on which the individual soil extracts were pooled together. To avoid potential pipetting errors, pools of three samples were first created (8 × 3-sample pools per plot), which were then used to produce pools of six samples (4 × 6-sample pools per plot) by combining equal quantities of two 3-sample pools. Pools of six were in turn mixed together to create pools of 12, which were finally combined to produce a pool of all 24 samples extracted from a plot. Following this method, a sum of 15 pooled samples were created per plot (alongside the 24 individual samples per plot) and 90 pooled samples were prepared in all along with 144 individual samples. A total of 234 eDNA extracts (both pooled and individual) then underwent the same following PCR amplification steps.

Based on amplification protocols outlined by the Earth Microbiome Project (Gilbert *et al.* 2014) (http://press.igsb.anl.gov/earthmicrobiome/protocols-and-standards/its/), two single-indexed DNA libraries were assembled from the 188 individual soil extracts and 90 pooled soil extracts using the fITS7 general fungal primer (5′- GTG ART CAT CGA ATC TTT G -3′) (Ihrmark *et al.* 2012) and the ITS4 reverse primer (5'- TCC TCC GCT TAT TGA TAT GC -3') (White *et al.* 1990). The ITS4 reverse primer was designed with both Illumina adapter sequences and index sequences (Caporaso *et al.* 2011), permitting future identification of the sequenced amplicons. The fITS7 primer (along with its Illumina adapter sequence) was ordered from "Integrated DNA Technologies" (Purification method: Standard Desalting). The Illumina adapter for fITS7 was 5'- AAT GAT ACG GCG ACC ACC GAG ATC TAC AC -3′ and the Illumina adapter for ITS4 was 5'- CAA GCA GAA GAC GGC ATA CGA GAT -3′.

The amplification experiments were performed using an Eppendorf vapoprotect Mastercycler® in a 25 µL mixture volume containing 0.2 µL FastStart™ DNA polymerase (Merck), 0.5 µL dNTP mixture (10 mM each), 2.5 µL PCR buffer (with 20 mM $MgCl_2$, sourced from Merck), 2 µL 2.5 µM of each forward and reverse primer, 1.25 µL 10 µM molecular grade Bovine Serum Albumin, 1 µL 10× diluted DNA template (same dilution for both pooled and individual soil eDNA extracts) and 15.55 µL filtered deionized water (obtained via Milli-Q® water purification system and filtered through a Biopak® Polisher). Bovine Serum Albumin was used to reduce the effect of PCR inhibitors derived from soil (Jiang *et al.* 2005). All PCR reagents prior to adding the DNA template were assembled in a dedicated UV light irradiated chamber with a dedicated set of micropipettes. PCRs were carried out under the following conditions: a denaturation step of 5 min at 94°C, followed by 35 cycles of 30 s at 94°C, 30 s at 57°C and 30 s at 72°C, with a final step at 72°C for 7 min (and held at 4°C). All PCRs were carried out in duplicate along with positive and negative controls. Agarose gel electrophoresis, stained with RedSafe™ (iNtRON) and using a 1% agarose gel, was performed on the PCR product to confirm amplification. No DNA was observed in negative controls and samples which showed poor amplification were rerun. The 10× dilution of the DNA templates (with filtered deionized water) was undertaken after initially performing PCRs

with 1 µL of the undiluted PowerSoil® DNA elution (both for pooled and individual samples), which yielded less PCR product according to gel runs, possibly due to PCR inhibition caused by soil components such as tannins (Kreader 1996). As the PCRs were performed in duplicate, 20 µL of each duplicate PCR product were combined prior to normalization.

Following manufacturer's instructions, SequalPrep™ Normalization Plates (ThermoFisher Scientific) were used to both clean the obtained amplicons and to normalize their concentration relative to each other. Twenty-five microlitres of mixed duplicate PCR product (25 µL being the recommended maximum) underwent normalization as part of the SequalPrep™ protocol. I eluted my normalized PCR product with 12 µL elution buffer (instead of 20 µL recommended by the manufacturer) due to a previous unsuccessful sequencing library submission that might possibly have been caused by too low a concentration of PCR amplicons. The resulting concentration of normalized samples was 2.14 ng/µL. Two sequencing libraries were prepared; for each library, 12 µL of normalized indexed PCR amplicons were pooled together, vortexed and 110 µL each of the two resulting mixtures were sent to Massey Genome Service, New Zealand, to undergo Illumina MiSeq™ (run option: 2×250 base PE v2) (Caporaso *et al.* 2012).

Bioinformatics and statistical analysis

I merged forward and reverse Illumina reads using a 32-bit version of USEARCH v11.0.667 (Edgar 2010). I removed any sequences with less than 200bp or which had more than one expected error using VSEARCH 2.10.4 (Rognes *et al.* 2016). In order to increase the qualitative nature of the sequencing reads and to account for PCR and sequencing artefacts (Leray and Knowlton 2017) and singletons (Dickie 2010), any sequences occurring either once or twice were removed, while the remaining sequences were clustered to 97% similarity threshold. Although both USEARCH and VSEARCH could have been used to filter sequences, the 64-bit version of VSEARCH is open-source and was therefore chosen. OTUs were matched using BLAST v2.5.0+ (Altschul *et al.* 1997) against the UNITE public database (accessed July 2019) (Nilsson *et al.* 2018). I removed all recorded OTUs which were not within the kingdom Fungi and all OTUs which had a <200 bp match to any known species. Extraction blanks, and positive and negative controls were checked for contamination and OTUs which were found within my negative controls (0.34% of all OTUs) were also deleted. In order to further limit the effects of PCR and sequencing artefacts (Vesty *et al.* 2017), I excluded low abundance OTUs by setting OTU occurrence to 0 if any OTU occurred less than 3 times in any sample. In the case where the same soil extract was sent to be sequenced twice (due to an insufficient amount of amplification observed via gel runs), the sample with the lowest number of reads was deleted along with any sample which had <1000 reads (0.23% of samples).

I used R version 3.5.0 (Team 2013) for creating graphs and conducting analyses. The subsample size for rarefying my community was set to the minimum number of sequences of any sample. I

created *in silico* samples which were pooled computationally to correspond with my physically pooled samples via the *aggregate* function in "stats (v3.62)" (Team 2013). When examining the composition of my samples according to fungal phylum, I generated randomly rarefied community versions of my dataset via the *rrarefy* function in "vegan" (Oksanen *et al.* 2013) before measuring the proportion belonging to a specific fungal phylum. This process was iterated 100 times and a mean proportion was calculated for each fungal phylum. The *rrarefy* function was here appropriate as random rarefaction is performed without replacement so that the variance of community metrics is not related to the size of the sample.

To quantify the effects of pooling on fungal rarefied richness and proportional abundance, I used linear mixed-effect models via the R package "lme4 (v1.121)" (Bates *et al.* 2014), setting sampling plot as a random effect. The mixed models break down the variance into inter- and intraplot components and thus improve the true structure of the randomness present in the data (Millar and Anderson 2004). When fitting my linear mixed-effect models to rarefied richness over *C. scoparius* % coverage, I optimised my model using the lme4's *update* function, reducing the model by excluding any interaction when the *P* value for the interaction term was higher than 0.05. I compared my simplified models to my original models using lme4's *anova* function to make sure that the interaction between both models was non-significant ($P > 0.05$).

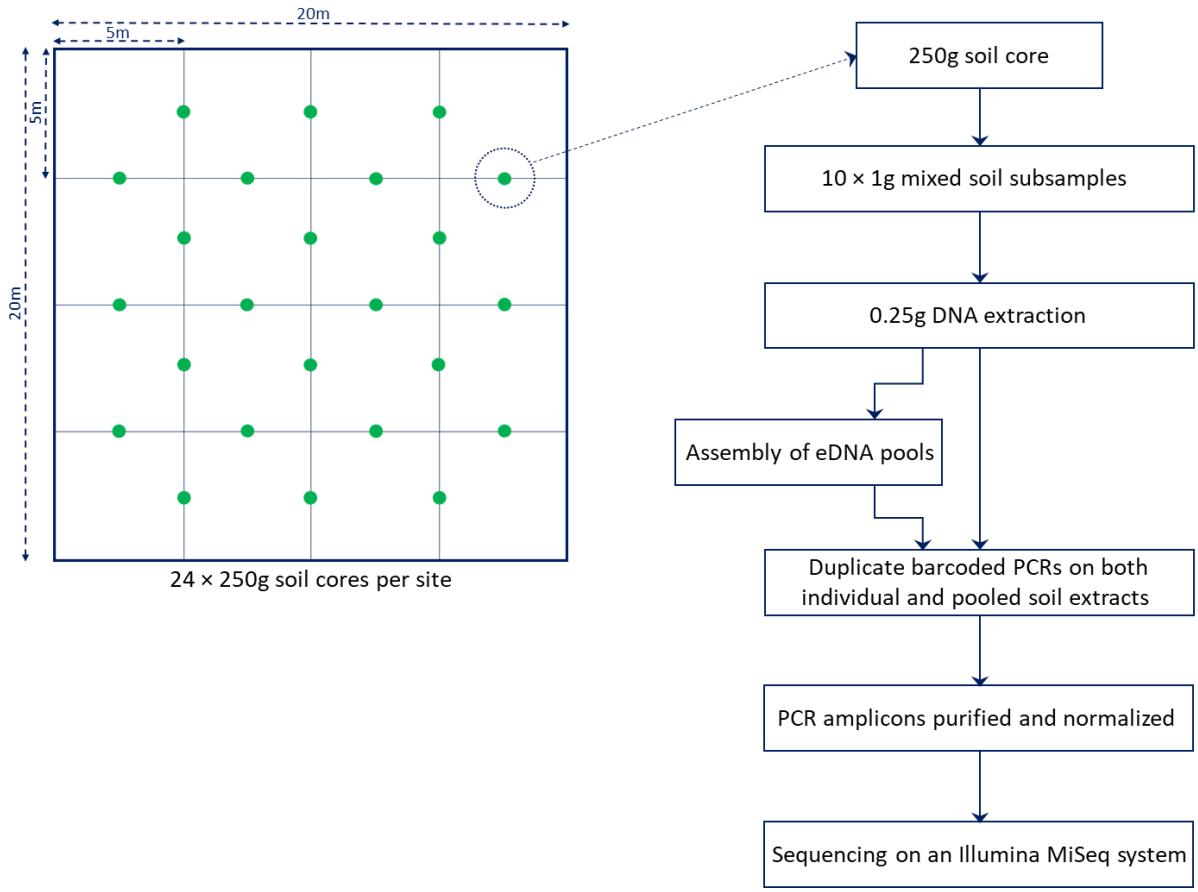


Figure 1. Overview of sample collection from each field plot and laboratory processes used in the study.

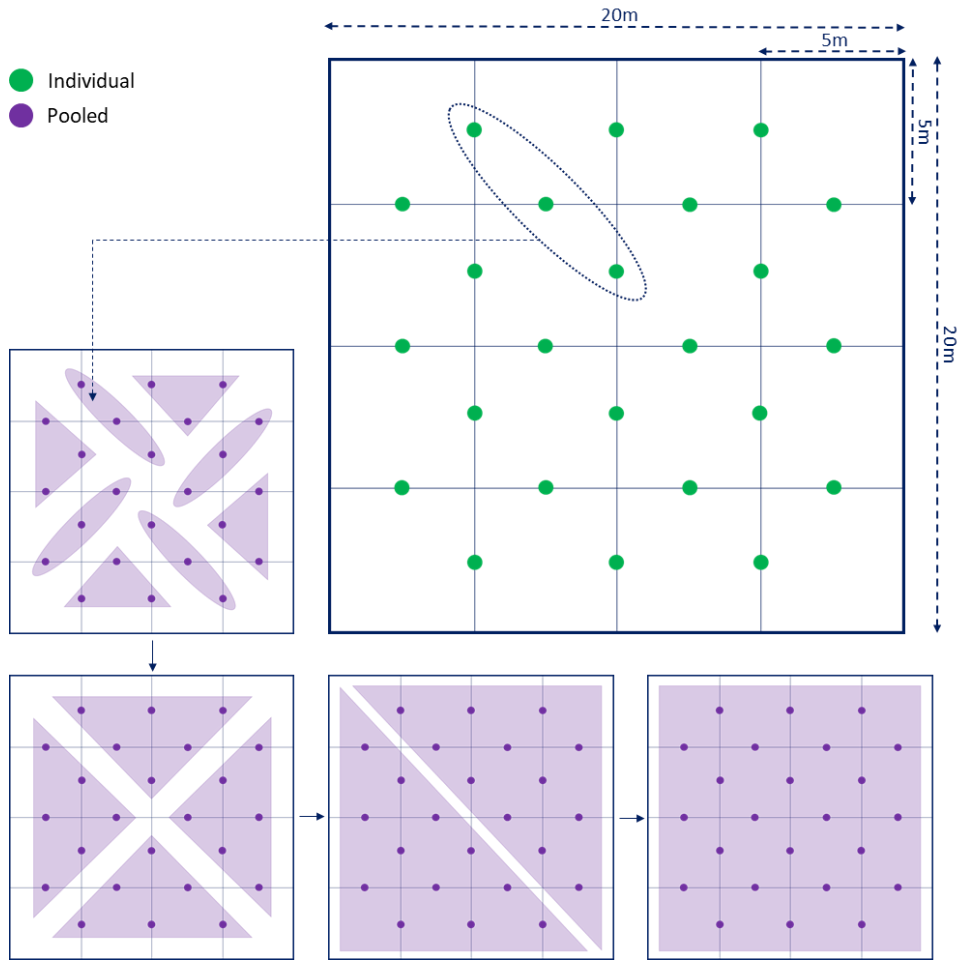


Figure 2. Partitions designed for the pooling of soil eDNA extracts. Individual cores are shown in green, pooled eDNA extracts comprised of the individual cores are shown in purple. Smaller pooling partitions were designed to fit into larger partitions.

Results

In total there were 3471 fungal OTUs, the three most dominant fungal phyla and subphyla being Ascomycota (2039 OTUs, 58.7%), Basidiomycota (1048 OTUs, 30.2%) and Mortierellomycotina (119 OTUs, 3.4%) (Appendix C1). Appendix C2 shows a summary of the mean number of OTUs according to fungal phyla and pooling treatment.

Effect of pooling on richness

Across all six plots, physcially pooling eDNA samples prior to PCR amplification increased the rarefied richness of pooled samples compared to individual samples, yet the rarefied richness was highest for each plot when the individual samples were computationally combined to correspond with the pooled samples (Figure 3). The higher the degree of physical pooling (i.e., the more stand-alone samples a pooled sample was initially composed of), the higher the loss in rarefied species richness when compared to individual samples pooled computationally. Compared with Basidiomycota and Mortierellomycotina, the loss in rarefied richness was most pronounced within the fungal phylum Ascomycota (Figure 4).

Effects of pooling on composition

Again across all six plots, pooling eDNA samples shifted the proportional abundance of three out of six tested fungal taxa, which consisted of the most dominant fungal phyla and subphyla in the dataset (Figure 5). Any degree of eDNA pooling resulted in a downwards shift in the proportional abundance of Ascomcota and augmented that of Basidiomycota and Mortierellomycotina. Pooling showed no apparent effect on the proportional abundance of Glomeromycotina, Chytridiomycota or Mucoromycotina, although a weak increasing trend in the proportional abundance of Mucoromycotina (t = 0.784, P = 0.0716) occurred when pooled. The calculations for proportional abundance were redone without rarefaction (flowchart in Appendix C7) and no qualitative difference in results was found.

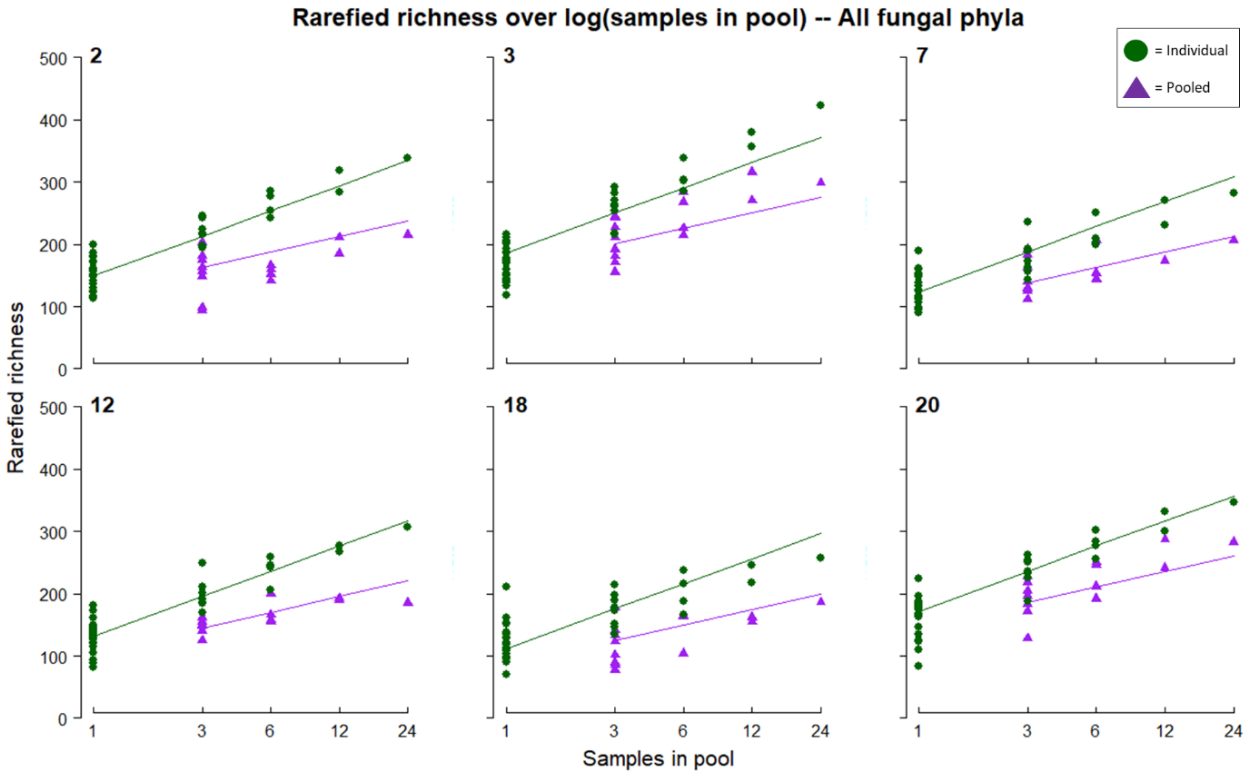
Fungal phyla which had a lower rank abundance in stand-alone eDNA samples (i.e., occurred in-field at relatively lower frequencies and/or were sequenced to a lesser degree), were less detectable the higher the degree of pooling (Table 1); yet the proportion of Mortierellomycotina OTUs increased the higher the degree of pooling (Appendix C8), which could also be observed for each of the 6 plots individually (Appendix C8). A similar general pattern was observed when looking at propotional rank sequence abundance both of all plots simultaneously (Appendix C9) and of each plot individually (Appendix C10). Whereas stand-alone samples were dominated by Ascomycota, when physically pooled, Basidiomycota and Mortierellomycotina both increased in rank sequence abundance. When considering the OTUs with the highest rank abundance within all individual

samples (n = 143), 9/10 were Ascomycota, yet only 2/10 of the most abundant OTUs were Ascomycota when analysing all pooled eDNA samples (n = 90).

Effects of pooling on perceived impact of C. scoparius

Cytisus scoparius coverage increased rarefied diversity for all fungi and for the three most abundant fungal phyla independently. Different levels of pooling showed dissimilar responses of rarefied fungal diversity to *C. scoparius* coverage (Figure 6), with higher levels of pooling showing a steeper upward trend for all fungi, for Basidiomycota and Mortierellomycotina, yet not for Ascomycota. When considering individual samples, *C. scoparius* coverage had little effect on the rarefied richness of Mortierellomycotina, yet when considering pooled samples, the rarefied richness of Mortierellomycotina increased with *C. scoparius* coverage (Figure 6).

As *C. scoparius* coverage increased, the rarefied richness of Basidiomycota, the 2nd most abundant fungal phylum, decreased in proportion to all fungal phyla when pooled computationally yet increased when pooled physcially (Figure 7). Also, as *C. scoparius* coverage increases, the proportion of Mortierellomycotina relative to all fungal phyla decreased except for when the samples were pooled by 12 or pooled by 24, in which case *C. scoparius* coverage has no considerable effect (Figure 7).



	Pooled	log(samples in pool)	Pooled × log(samples in pool)
Rarefied richness of all fungal phyla	**< 0.0001**	**< 0.0001**	**< 0.0001**
Rarefied richness of Ascomycota	**< 0.0001**	**< 0.0001**	**< 0.0001**
Rarefied richness of Basidiomycota	**< 0.0001**	**< 0.0001**	**< 0.0001**
Rarefied richness of Mortierellomycotina	**< 0.0001**	**< 0.0001**	**< 0.0001**

Figure 3. Rarefied richness over number of samples per pool (note log scale axis) for all fungal phyla. Purple triangles denote the physical pooling of samples whereas green circles represent the corresponding individual samples, the rarefied diversity of which has been pooled computationally to correspond with the physically pooled samples. Individual plot numbers are indicated on the top left of each graph and lines follow linear mixed-effect model fit. The p-value estimates in the linear mixed-effect model are presented in the below table (accompanying t-values are compiled in Appendix C3).

Figure 4. *[Next page]* Rarefied richness over number of samples per pool for individual fungal phylum. Purple triangles denote the physical pooling of samples whereas green circles represent the corresponding individual samples, the rarefied diversity of which has been pooled computationally to correspond with the physically pooled samples. Individual plot numbers are indicated on the top left of each graph and lines follow linear mixed-effect model fit. The p-value estimates in the linear mixed-effect model are presented in the above table (accompanying t-values are compiled in Appendix C3).

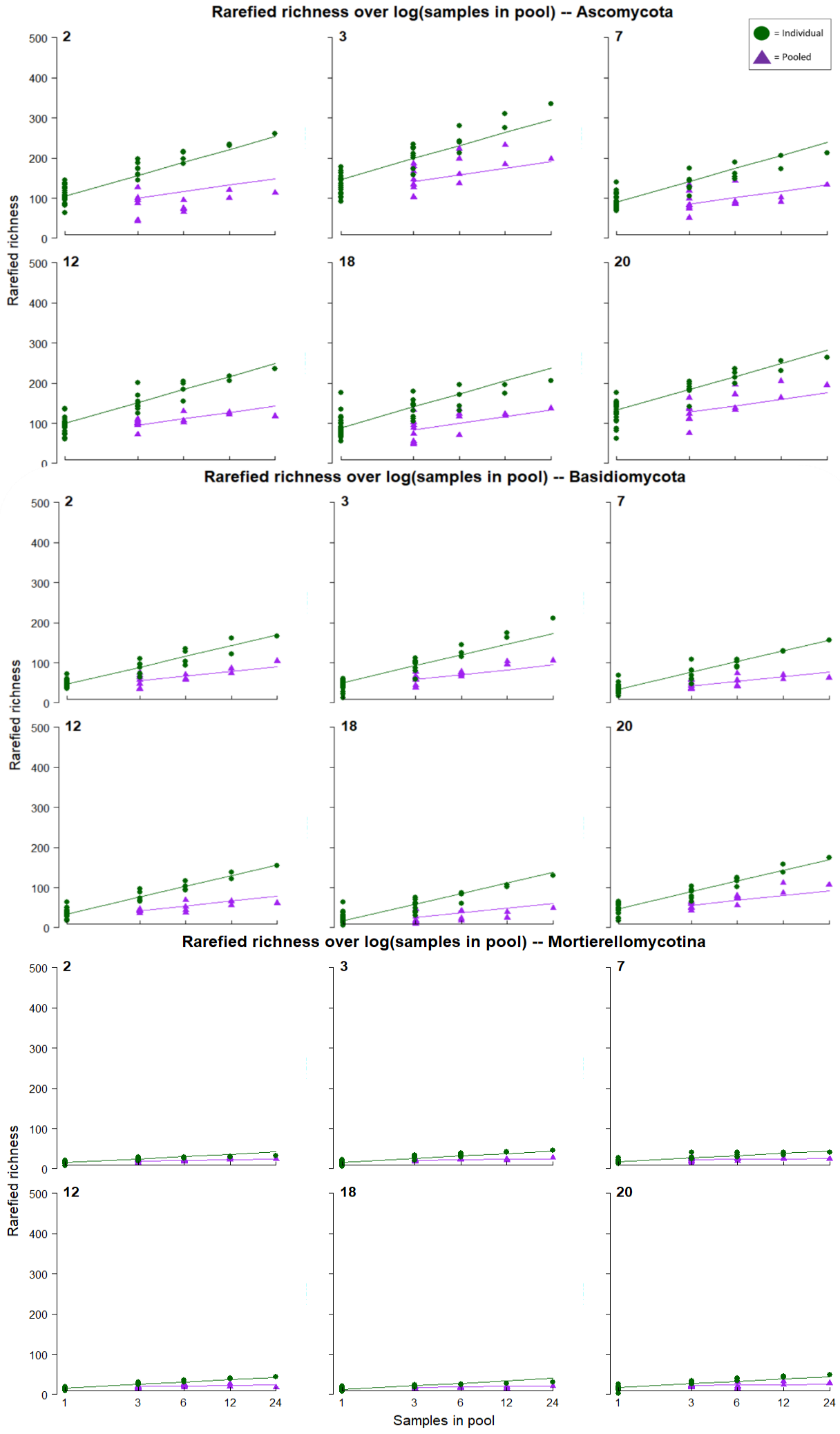
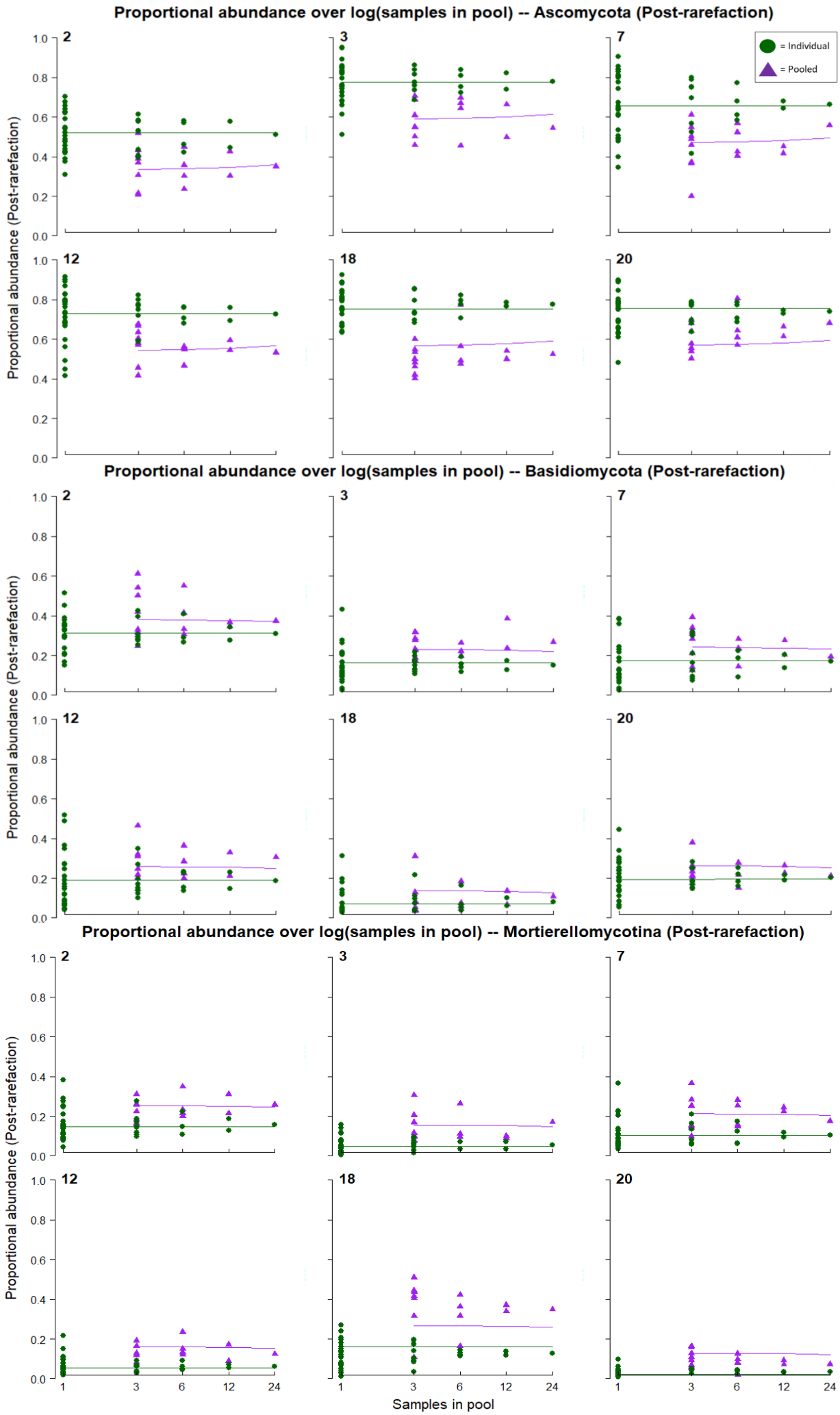

Rarefied richness over log(samples in pool) -- Ascomycota
= Individual
= Pooled
2
3
7
12
18
20
Rarefied richness
0
100
200
300
400
500
Rarefied richness over log(samples in pool) -- Basidiomycota
Rarefied richness over log(samples in pool) -- Mortierellomycotina
1
3
6
12
24
Samples in pool

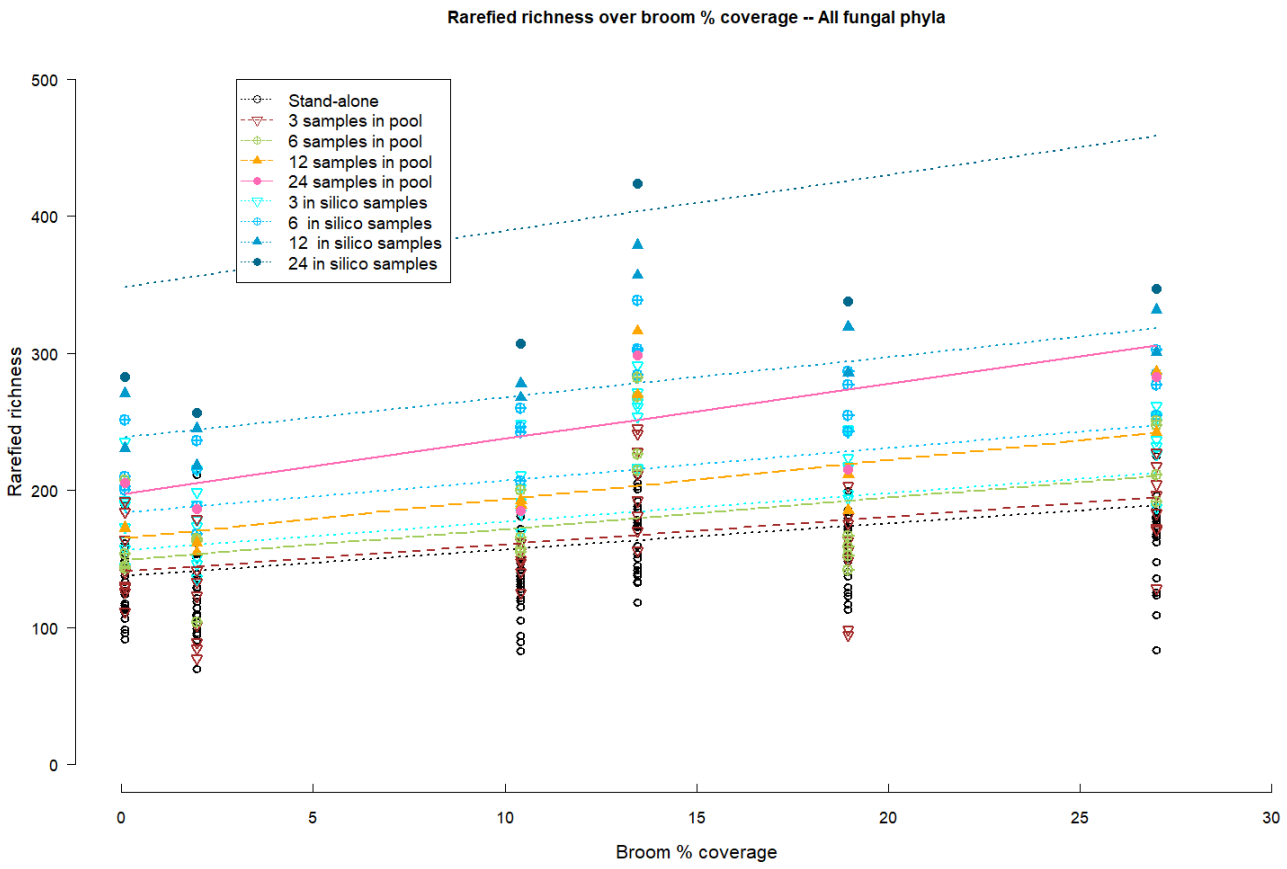
Figure 5. *[Next page]* Proportional abundance by fungal phylum over number of samples per pool (note log scale axis). Purple triangles denote the physical pooling of samples whereas green circles represent the corresponding individual samples, the proportional abundance of which has been pooled computationally to correspond with the physically pooled samples. Individual plot numbers are indicated on the top left of each graph and lines follow linear mixed-effect model fit. The p-value estimates in the linear mixed-effect model are presented in the above table (accompanying t-values are compiled in Appendix C4).

	Pooled	log(samples in pool)	Pooled × log(samples in pool)
Proportional abundance of Ascomycota	**< 0.0001**	0.7390	0.6559
Proportional abundance of Basidiomycota	**< 0.0001**	0.8656	0.8175
Proportional abundance of Mortierellomycotina	**< 0.0001**	0.8593	0.8123


Proportional abundance over log(samples in pool) -- Ascomycota (Post-rarefaction)
Proportional abundance over log(samples in pool) -- Basidiomycota (Post-rarefaction)
Proportional abundance over log(samples in pool) -- Mortierellomycotina (Post-rarefaction)
= Individual
= Pooled
2
3
7
12
18
20
Proportional abundance (Post-rarefaction)
Samples in pool
1
3
6
12
24
0.0
0.2
0.4
0.6
0.8
1.0

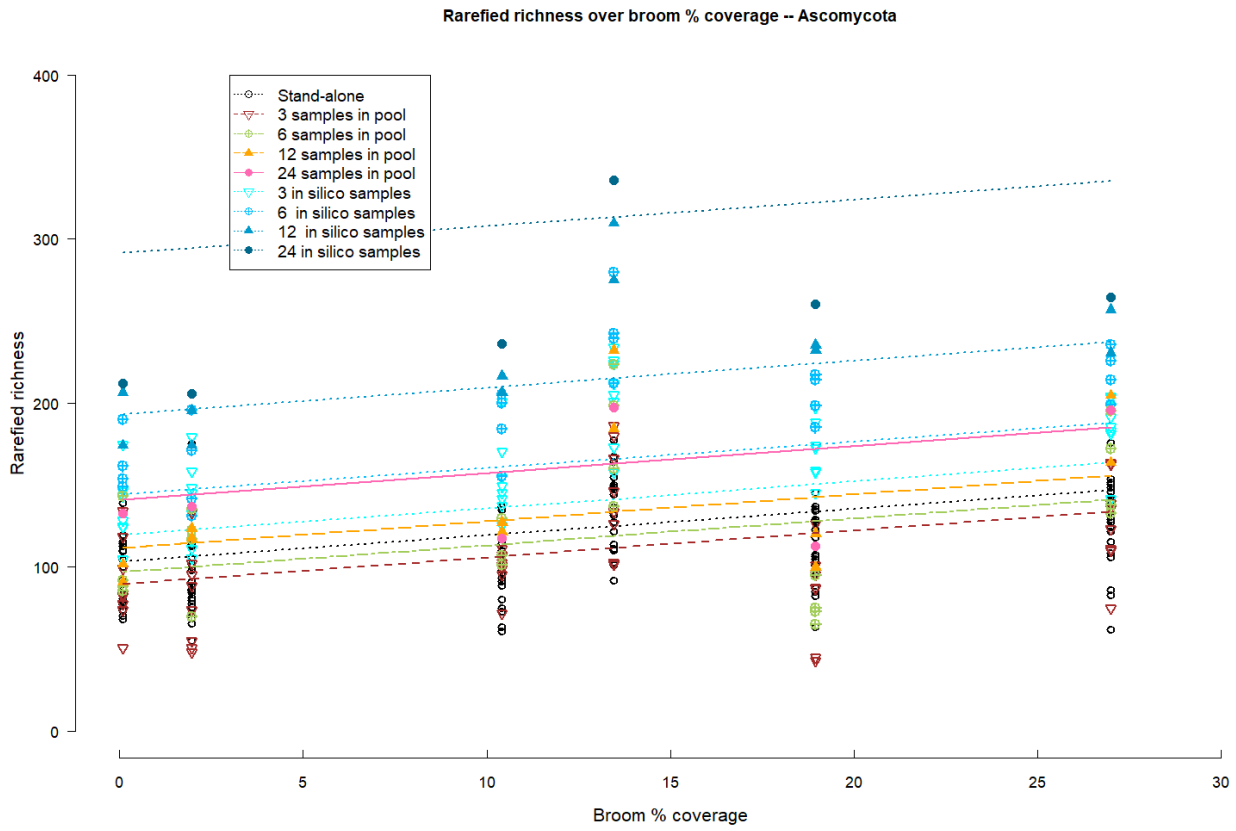
		Dominant	Core	Rare	**Total**
Stand-alone	Ascomycota	323	296	277	896
	Basidiomycota	124	134	146	404
	Chytridiomycotina	**0**	4	**0**	4
	Glomeromycotina	4	7	10	21
	Mortierellomycotina	21	22	20	63
	Mucoromycotina	8	12	21	41
	Unidentified	**0**	8	5	13
Pooled by **3**	Ascomycota	171	159	154	484
	Basidiomycota	59	66	68	193
	Chytridiomycotina	**0**	**0**	**0**	**0**
	Glomeromycotina	**0**	4	6	10
	Mortierellomycotina	12	9	14	35
	Mucoromycotina	3	5	**0**	8
	Unidentified	**0**	**0**	3	3
Pooled by **6**	Ascomycota	141	129	133	403
	Basidiomycota	49	55	54	158
	Chytridiomycotina	**0**	**0**	**0**	**0**
	Glomeromycotina	**0**	4	5	9
	Mortierellomycotina	11	9	7	27
	Mucoromycotina	**0**	4	3	7
	Unidentified	**0**	**0**	**0**	**0**
Pooled by **12**	Ascomycota	125	107	119	351
	Basidiomycota	38	49	46	133
	Chytridiomycotina	**0**	**0**	**0**	**0**
	Glomeromycotina	**0**	**0**	3	3
	Mortierellomycotina	9	10	3	22
	Mucoromycotina	**0**	4	3	7
	Unidentified	**0**	**0**	**0**	**0**
Pooled by **24**	Ascomycota	89	85	79	253
	Basidiomycota	28	34	32	94
	Chytridiomycotina	**0**	**0**	**0**	**0**
	Glomeromycotina	**0**	**0**	**0**	**0**
	Mortierellomycotina	7	5	8	20
	Mucoromycotina	**0**	**0**	3	3
	Unidentified	**0**	**0**	**0**	**0**

Table 1. Taxonomic composition of OTUs across all plots, split into the top 3rd ("dominant"), middle 3rd ("core") and bottom 3rd ("rare") proportional rank abundance percentile for each degree of pooling. Note that "0" values are in bold to accentuate the loss of rare fungal taxa the higher the degree of pooling. The same data is visualised in Appendix C8 in terms of proportions.



	Broom coverage	Samples in pool	Pooled	Broom coverage × samples in pool	Samples in pool × pooled
Rarefied richness of all fungal phyla	**0.02558**	**< 0.0001**	**< 0.0001**	**0.02870**	**< 0.0001**
Rarefied richness of Ascomycota	0.06400	**< 0.0001**	**< 0.0001**	.	**< 0.0001**
Rarefied richness of Basidiomycota	**0.01834**	**< 0.0001**	**< 0.0001**	**0.00981**	**< 0.0001**
Rarefied richness of Mortierellomycotina	0.44781	**< 0.0001**	**< 0.0001**	**0.01559**	**< 0.0001**

Figure 6. Rarefied richness over mean *C. scoparius* % coverage for all fungal phyla (above) and for individual fungal phylum (next page). Lines follow linear mixed-effect model fit. The p-value estimates in the linear mixed-effect model are presented in the above table (accompanying t-values are compiled in Appendix C5).



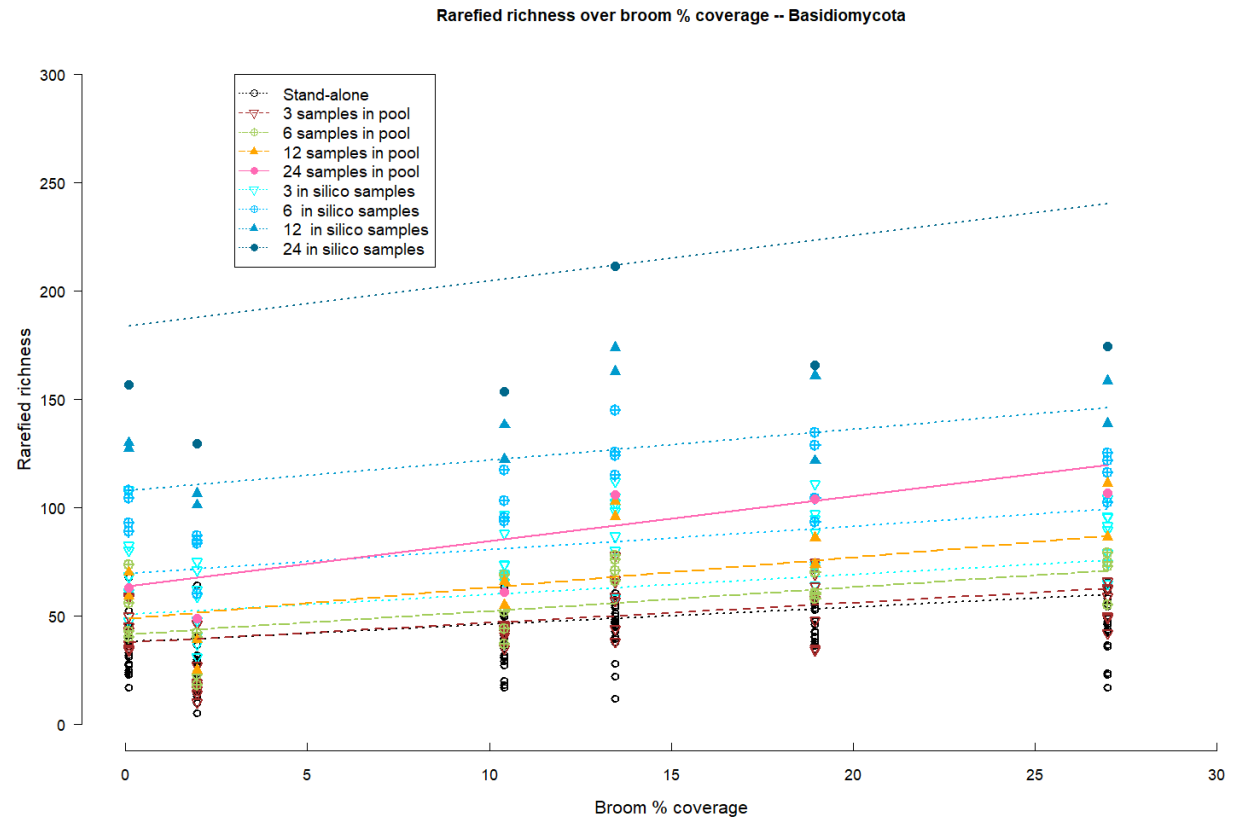


Figure 6. *[Continued]*

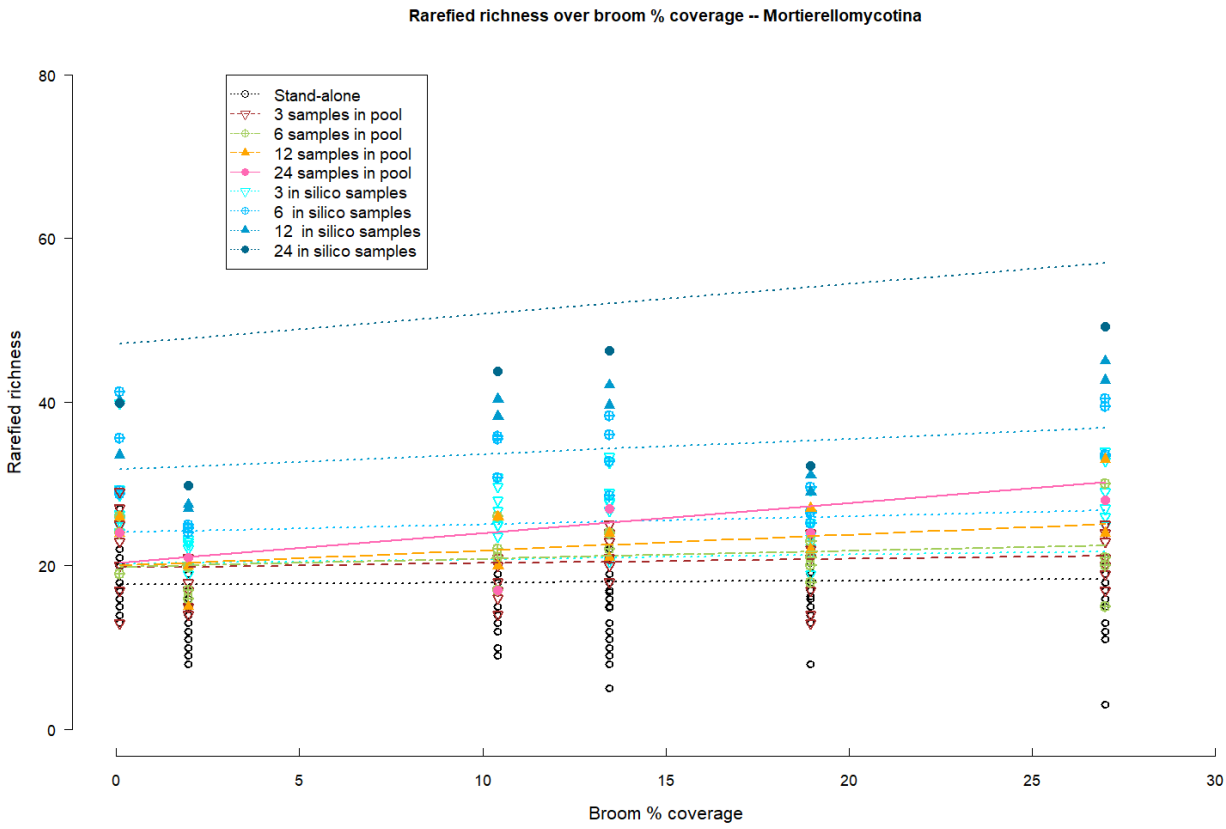
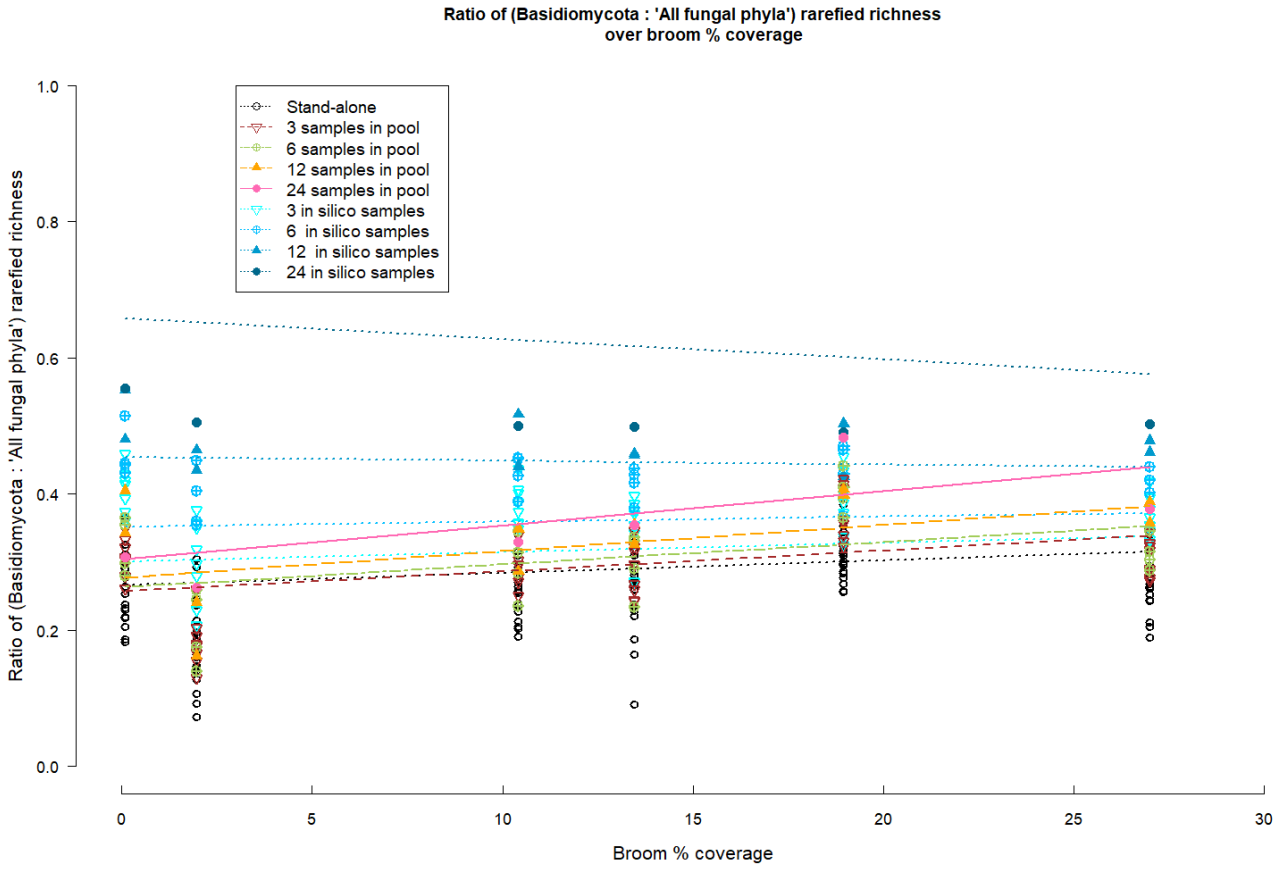
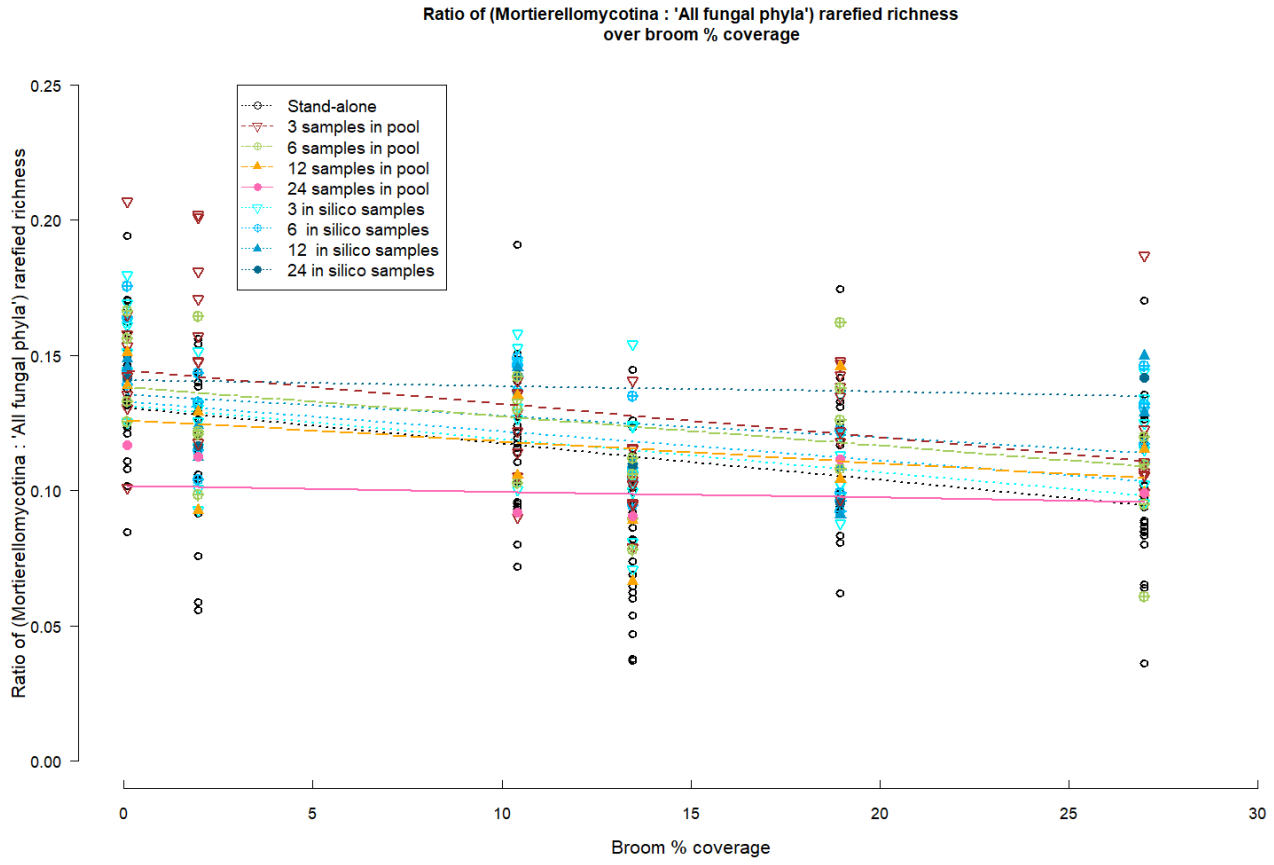


Figure 6. *[Continued]*



Rarefied richness of	Broom coverage	Samples in pool	Pooled	Broom coverage × samples in pool	Broom coverage × pooled	Samples in pool × pooled	Broom coverage × samples in pool × pooled
Basidiomycota ~ of all fungal phyla	0.294	**< 0.0001**	**< 0.0001**	0.205	**0.00382**	**< 0.0001**	**0.0372**
Mortierellomycotina ~ all fungal phyla	**0.04234**	0.80651	**0.01272**	0.09242	.	**< 0.0001**	.
Ascomycota ~ all fungal phyla	0.9277	0.1055	**< 0.0001**	.	.	.	.

Figure 7. Rarefied richness of individual fungal phyla in proportion to the rarefied richness of all fungal phyla over mean *C. scoparius* % coverage. Lines follow linear mixed-effect model fit. The p-value estimates in the linear mixed-effect model are presented in the above table (accompanying t-values are compiled in Appendix C6).



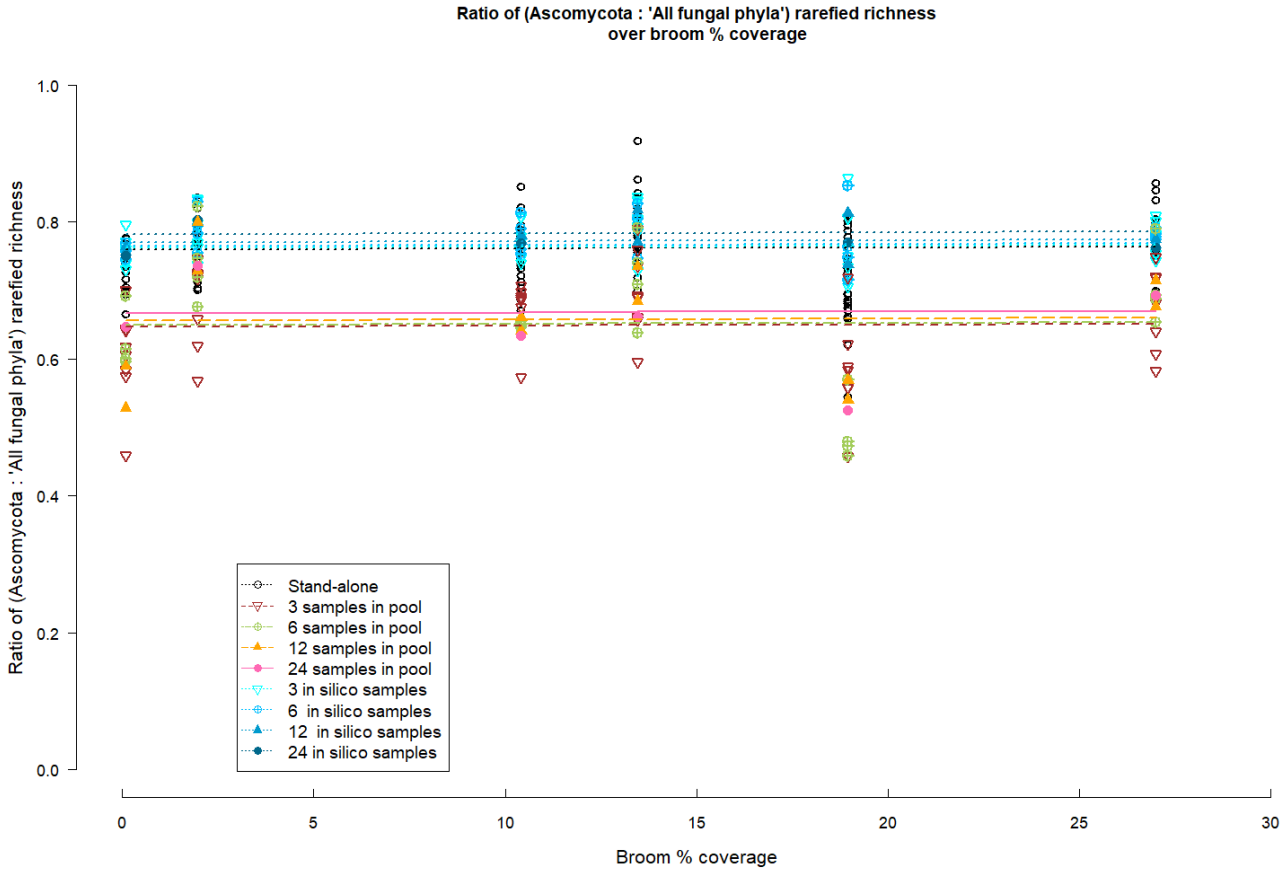


Figure 7. *[Continued]*

Discussion

The results of this study suggest that, compared with computationally pooled soil eDNA extracts, physically pooling soil eDNA pre-PCR will 1) decrease observable fungal rarefied richness, 2) lead to fungal phylum-specific shifts in proportional abundance and 3) increase the sensitivity regarding how an invasive plant's overarching impact on fungal diversity is detected. Pooling fungal eDNA might change the outcome of similar eDNA studies where the aim is 1) to identify the rare biosphere within a soil community, 2) to estimate species richness and proportional abundance, or 3) to assess the impact of an invasive plant on soil fungi. In more detail, compared with computationally pooled soil eDNA extracts, physically pooling soil eDNA pre-PCR will 1) decrease observable fungal rarefied richness, particularly for Ascomycota in relation to Basidiomycota and Mortierellomycotina, 2) lead to fungal taxa-specific shifts in proportional abundance, which increases for Basidiomycota and Mortierellomycotina at the expense of Ascomycota, and 3) increase the sensitivity as to what extent an encroaching invasive plant (*C. scoparius*) increases the rarefied richness of all fungi as well as the richness of individual fungal taxa. Moreover, the effect of *C. scoparius* on the relative rarefied richness of Basidiomycota (in proportion to all fungi) can be influenced by whether or not an eDNA study uses sample pooling.

Effect of pooling on richness

Soil commonly harbours a large diversity of microorganisms in close proximity as a function of physical characteristics (Kang and Mills 2006). As shown in my results on rarefied richness over the number of samples per pool, it was therefore expected that combining multiple samples captures more OTU richness compared to individual samples (Song *et al.* 2015). Even though this increase in rarefied richness was expected, physically pooled fungal eDNA has been known to have a lower fungal species richness when compared to individual samples (Branco *et al.* 2013), despite a pooled sample encompassing a larger area. This decrease in species richness is due to rare taxa being not well-represented in pooled samples (Ohman and Lavaniegos 2002) and my results show that minor levels of physical pooling (3 stand-alone samples per pool) risk reducing the rarefied richness of Ascomycota in particular.

Effects of pooling on composition

Given the shifts in proportional abundance observed in my data, where pooling decreased the proportional abundance of the community's most dominant fungal phylum (Ascomycota) in favour of less common taxa (Basidiomycota and Mortierellomycotina), it may be proposed that individual OTUs of Ascomycota are spatially rare but locally dominant. On the other hand, OTUs of Basidiomycota and Mortierellomycotina occur more homogenously distributed in a given area,

yet with lower dominance (a general overview of the occupancy of individual fungal OTUs by phyla is given in Appendix C11). Although restricted to a broad observation, such differing fungal spatial distributions could be responsible for why pooling stand-alone eDNA rich in locally dominant Basidiomycota would dilute the more homogeneous Ascomycota, thus making them less detectable by metabarcoding techniques as well as increasing the proportional abundance of Basidiomycota while decreasing that of Ascomycota.

Effects of pooling on perceived impact of C. scoparius

The significant interaction of *C. scoparius* cover and perceived species richness suggests that pooling is a useful technique when studying overarching large-scale effects such as the impacts of an invasive plant on soil communities, as suggested by Ellingsøe & Johnsen (2002). Pooling reduced the variability between samples, thereby providing a more general expression of the overall community structure in a given plot (Ellingsøe and Johnsen 2002, Manter *et al.* 2010), yet at the expense of within-plot accuracy obtained by using a higher number of samples (Ranjard *et al.* 2003). When considering how using pooled samples showed an increase in the proportion of Basidiomycota (relative to all fungi) as *C. scoparius* increases whereas stand-alone samples showed a decrease, the question remains as to which is the 'real' process taking place, yet it is evident that dissimilar (and in this case mutually exclusive) broad ecological effects can be observed according to sample processing.

Experimental design considerations

There are multiple biases surrounding PCR (Acinas *et al.* 2005) and regulating the amount of DNA template for PCR has been known to improve PCR efficiency (Wilson 1997, Lindahl *et al.* 2013). Although all PCRs were performed in duplicate to reduce bias in template-to-product ratios (Polz and Cavanaugh 1998), the methods do not take into account how the concentration of the soil eDNA pre-PCR might have impacted the composition of the physical pools (e.g., some soil extracts could have had a much lower soil eDNA concentration from the offset). I did not quantify the soil eDNA extract as it would have been very difficult to determine how much of the extracted DNA was derived from fungi, yet observing similar patterns across six plots should account for any differences in the relative concentration of my pooled samples pre-PCR.

I pooled DNA extracts rather than soils. Instead of extracting DNA from 0.25g of soil per collected sample, if I had instead sampled 0.25g of soil from a pool of multiple (i.e., up to 24) soil cores, this would almost certainly have caused a very large variability between samples which I wanted to avoid.

Conclusions and applications

Pooling is a common practice in eDNA studies (Dickie *et al.* 2018), both at the level of plot (Osborne *et al.* 2011) or within subset categories such as soil depth (Tveit *et al.* 2013). Although there have been two notable cases where pooling before or after PCR was observed to have little effect on the perceived community (Manter *et al.* 2010, Osborne *et al.* 2011), in both cases this was specific to bacteria as opposed to fungi and it has been suggested that fungi can be more susceptible to pooling due to having a more spatially heterogeneous distribution compared to bacteria (Manter *et al.* 2010). Such a patchy fungal spatial heterogeneity, which has long been observed at fine scales (Horton and Bruns 2001), could be an underlying factor causing locally dominant but spatially rare taxa to become overly diluted in pooled eDNA samples, rendering them untraceable to metabarcoding techniques.

The decision whether or not to pool can be highly context-dependent and relies on weighing up costs of field sampling, DNA extraction, wet-lab processing and the sequencing technology (or other approach) used to identify the samples. The spatial heterogeneity of the studied organisms as well as the trade-offs between increased replication and improved precision per replicate need to be likewise taken into account (Dickie *et al.* 2018). It is uncommon in eDNA studies that multiple stand-alone samples are taken within a statistical replicate (Dickie *et al.* 2018), even so, such an approach should be encouraged if both within and between plot variability in community composition is to be examined (Drummond *et al.* 2015, Navarrete *et al.* 2015).

When considering the possible benefits of pooling, particularly in relation to how *C. scoparius* coverage in my dataset increases fungal rarefied richness, it is important to note that the intra-plot variance which pooling decreases is not necessarily 'distracting noise' (Ranjard *et al.* 2003), but potentially valuable ecological information. However, if the goal of a study is to test large scale patterns, unexplained intra-plot variance can be an obvious hurdle. With this in mind, I have two recommendations regarding the use of eDNA pooling:

- If the objective of an eDNA survey is study the rare biosphere or the proportional abundance of fungi in a given environment, then my results support Lear *et al.* (2018)'s recommendation that sample pooling should be avoided in favour of analysing more small subsamples and it can be added that this is particularly applicable if the fungal phyla of interest is Ascomycota, Mucoromycota, Glomeromycotina or Chytridiomycota.

- If however the objective of an eDNA survey is to study a large scale overarching effect across several plots such as that of an invasive plant species on fungal communities, then my results support Ellingsøe and Johnsen (2002)'s recommendation to use larger sample sizes, or in my case pooled samples, as these reduce intra-plot variation allowing broader-

scale effects to override the abundant fine-scale variation present in fungal communities (Dickie *et al.* 2002, Tedersoo *et al.* 2003, Taylor *et al.* 2014).

I would add that pooling is most amenable to Mortierellomycotina and Basidiomycota and can still be a cost-effective way to calculate species richness across larger areas, but is not as effective as multiple stand-alone samples. A future research avenue would be to examine the potential of eDNA pooling as a means of cost-effectively detecting changes in soil fungal composition caused by large-scale events such as anthropogenic activity or the spread of an invasive species.

Chapter 5: The fungal component of the soil legacy of *Cytisus scoparius*

Abstract

Soil legacy effects can be an important contributing cause of plant rarity and invasiveness in communities. However, the identity and composition of soil biota which may underlie plant growth responses and nutrient acquisition are rarely accounted for, even though soil microbiota can determine the availability of many essential plant nutrients or adversely stunt plant growth through antagonism, among other effects. I aimed to examine whether the soil legacy of *Cytisus scoparius* (Fabaceae) could be explained in terms of fungal communities. I used soil with known fungal community composition extracted from across a density gradient of exotic *C. scoparius* to test whether the fungal component of the soil legacy of *C. scoparius* favoured the growth and nutrient acquisition of a selection of plants native and exotic to New Zealand which were either able or unable to fix nitrogen. I found that of all tested fungal predictors, increased arbuscular mycorrhizal fungi richness induced by the soil legacy of *C. scoparius* favoured the growth of Fabaceae, particularly exotic Fabaceae, compared with non-N-fixing native plants. My results suggested that exotic Fabaceae may form interaction with a higher richness of arbuscular mycorrhizal fungi than native plants, which may underlie their invasiveness.

Keywords

Arbuscular mycorrhizal fungi, *Cytisus scoparius,* environmental DNA, functional traits, fungal communities, metabarcoding, mutualisms, soil legacy

Introduction

Plant-soil feedback, the process whereby a plant's effect on soil biotic and abiotic properties influences the growth of future generations of plants, is generally considered to be an important contributing cause of plant rarity and invasiveness in communities (Klironomos 2002). In Chapter 2, I performed a greenhouse experiment examining the effects of soil under various levels of an invasive leguminous shrub (*Cytisus scoparius*). For that greenhouse experiment, I dug up soil from 18 permanent vegetation plots across a natural *C. scoparius* density gradient. The vegetation of the 18 plots ranged from grassland uninvaded by *C. scoparius* to near-monocultures of *C. scoparius*. I then cultivated 16 plant species in the 18 collected soils for ~7 months in a greenhouse environment (total number of plants = 288). At harvest, I measured shoot height, root and shoot dry biomass and obtained measurements for shoot % N and shoot % P. After plant morphometric and nutrient responses were analysed with accompanying data on soil chemistry from each of the 18 sampling plots (e.g., Olsen P, Ca, Mg), the results suggested that although some soil chemical traits had a slight correlation with the effect of *C. scoparius* coverage on plant growth, a portion of the effect of *C. scoparius* coverage remained unexplained (Table 4; Chapter 2).

Soil microorganisms often have significant positive and negative effects on plants through root-rhizosphere mutualism (Brundrett 1991), pathogen effects (Burdon 1993, Packer and Clay 2000) and by driving nutrient cycles (Crowley *et al.* 1991). In Chapter 3, I set out to measure part of the biological effect of *C. scoparius* by undertaking a natural survey on how fungal communities surrounding *C. scoparius* differed according to varying degrees of *C. scoparius* invasion. I sampled soil from the same plots which I used for my greenhouse experiment (Chapter 2) and determined that *C. scoparius* caused an unexpected increase in the diversity of several fungal taxa and functional groups (summarized in Table 2; Chapter 3).

Soil legacy studies are typically performed by studying plant growth in soil moulded by a previous plant (e.g., Grove *et al.* (2015)) or soil which has undergone a particular treatment (e.g., Pernilla Brinkman *et al.* (2010)). Although measuring soil chemical attributes is relatively common in soil legacy studies, the identity and composition of soil biota which may underlie plant growth responses (or nutrient acquisition) are rarely accounted for, even though soil microbiota can determine the availability of many essential plant nutrients (Edwards *et al.* 2019, Wilschut *et al.* 2019) or adversely stunt plant growth through antagonism (Latz *et al.* 2016, Schroeder *et al.* 2020), among other effects.

One of the most important functional groups of soil biota for plant growth is arbuscular mycorrhizal fungi (AMF), which play a key role in P uptake. P deficiency has long been known to

negatively affect plant growth (Mengel and Kirkby 1982). Although P is present in the biosphere at high concentrations, plants primarily directly absorb orthophosphate (Pi). Pi is a form scarce in soil (Raghothama 1999) and also essential for microbial growth (Richardson and Simpson 2011, Zhu *et al.* 2016). Apart from improving plant P acquisition (Collins and Foster 2009), AMF may also contribute to defence against pathogens (St-Arnaud and Vujanovic 2007) and increased abiotic stress tolerance (Augé *et al.* 2015). The effect of AMF on plant growth can vary widely depending on both plant taxa (Koch *et al.* 2017) and whether or not associating AMF are native or exotic (Klironomos 2003). In terms of plant P uptake, different species of AMF likewise differ in their efficiency (Miransari *et al.* 2009). When faced with multiple abiotic stress factors, it is known that plants can benefit in terms of increased biomass and mineral nutrition when grown in soil inoculated with multiple species of AMF compared with soil containing only a single species of AMF (Higo *et al.* 2018, Crossay *et al.* 2019, Crossay *et al.* 2020).

Knowing 1) how the soil legacy of *C. scoparius* affects plants with differing natural traits (Chapter 2), and 2) the change in fungal community composition entwined in the soil legacy of *C. scoparius* (Chapter 3), I aimed to examine whether the predominantly positive soil legacy of *C. scoparius* could be explained in terms of fungal communities.

I hypothesised that:

- One or several changes in fungal diversity induced by *C. scoparius* (Chapter 3) drives the mostly positive soil legacy effect of *C. scoparius* (Chapter 2).

- One or several changes in fungal proportional abundance induced by *C. scoparius* drives the mostly positive soil legacy effect of *C. scoparius*.

Methods

The starting point relating to my greenhouse data was my raw data on plant biomass and shoot % N and shoot % P which I obtained in Chapter 2, excluding plants found dead at harvest (3.8% of total).

I chose to focus on the fungal diversities within my individual soil eDNA samples (n = 431) as opposed to my pooled eDNA samples, as my pooled eDNA samples only deal with a subset of the 18 plots used in my natural survey. I used R version 3.5.0 (Team 2013) for creating graphs and conducting analyses. All fungal diversity and proportional abundance estimates (e.g., Ascomycota rarefied richness) were based on the same evenly rarefied OTU matrices which I used in Chapter 3 and the subsample size for rarefying my community was set to the minimum number of sequences in any sample. When examining the composition of my samples according to fungal taxon and functional guild, I first generated randomly rarefied community versions of my dataset via the *rrarefy* function in "vegan" (Oksanen *et al.* 2013). This process was iterated 250 times before calculating mean rarefied richness and proportional abundances for each fungal taxon and functional guild across all iterations. The *rrarefy* function was here appropriate so that community metrics are not biased by the size of the sample.

To quantify the effects of the soil legacy of *C. scoparius* on plant biomass and nutrient composition according to plant species traits, I used linear mixed-effect models via the R package "lme4 (v1.121)" (Bates *et al.* 2014) to account for plant species and plot. I performed model simplification based on *update* in the "lme4 (v1.1)" package to narrow down which response variables (if any) best predicted changes in plant biomass, shoot % N and shoot % P. Following the procedure described in Crawley (2002), third-order interactions (or higher) were only considered significant when $P < 0.01$, while main effects and second-order interactions were considered significant at $P < 0.05$. All tested continuous variables were scaled when performing mixed-effect models. In order to avoid over-parameterisation of models, I undertook two consecutive steps regarding mixed-effect modelling:

1) What are the drivers of plant growth?

Setting plant species and soil sampling plot as random effects, I tested the effect of all estimates of fungal rarefied richness and proportional abundance per plot for those fungal groups which had shown a significant ($P < 0.05$) response to *C. scoparius* at the plot scale in Chapter 3 (Table 2; Chapter 3) against shoot biomass, shoot % N and shoot % P (from Chapter 2).

2) Do the identified drivers of plant growth vary according to plant functional traits?

I then tested whether the explanatory variables obtained in the above step could "fit into" the original model used in my greenhouse experiment. In this original model, I found a significant three-way interaction for shoot biomass between *C. scoparius* coverage, plant origin, and legume status. Plant species and soil sampling plot were set as random effects while plant origin (i.e., 'Native' or 'Exotic') and legume status (i.e., 'Fabaceae' or 'Non-Fabaceae') were set as fixed effects.

Results

What are the drivers of plant growth?

Several explanatory variables were retained in the best model for shoot biomass, shoot % N and shoot % P (Table 1 relating to fungal richness; Table 2 relating to fungal proportional abundance). Distance to *C. scoparius* and both the proportional abundance and rarefied richness of Glomeromycotina (i.e., AMF) had an effect ($P < 0.05$) on shoot biomass whereas the rarefied richness of plant pathogens and saprotrophs both affected shoot % N and shoot % P. There was a positive correlation between AMF rarefied richness, AMF proportional abundance and an indicator for *C. scoparius* coverage (Appendix D1), although it was noted that one of the 18 soil plots had a very high AMF proportional abundance compared to the rest.

Do the identified drivers of plant growth vary according to plant functional traits? – Shoot biomass

Once the chosen explanatory variables (Tables 1 & 2) were added to a model which included plant origin and legume status as fixed effects, a three-way significant interaction was found for shoot biomass between AMF rarefied richness, plant origin, and legume status ($t = -4.482$; $P < 0.0001$; Table 3). Although increased AMF richness caused an overall increase in shoot biomass regardless of plant taxonomy or plant functional traits, AMF richness most notably increased the shoot biomass of the exotic Fabaceae *Trifolium repens* and *Ulex europaeus* (Figure 1).

Do the identified drivers of plant growth vary according to plant functional traits? – Shoot % N and % P

A three-way significant interaction was found for shoot % N between saprotroph richness, plant origin and legume status ($t = 2.841$; $P = 0.0045$; Table 3). When looking at each plant species individually, no discernible effect of saprotroph richness was observed on shoot % N (Appendix D2).

No three-way interaction was observed for shoot % P, although a two-way interaction was observed for shoot % P between plant pathogen richness and legume status ($t = 2.411$; $P = 0.0159$; Table 3) as well as saprotroph richness and legume status ($t = -2.209$; $P = 0.0272$; Table 3). When looking at each plant species individually, there was no obvious effect of saprotroph or pathogen richness on shoot % P (Appendix D3).

Table 1. Linear mixed-effect model *P* value extimates and effect sizes for all species rarefied richness predictors and reponse variables which previously showed a significant response to *C. scoparius* at the plot-scale. Plant species and soil sampling plot were both set as random effects. "**.**" indicates term dropped during model simplification. Distance to *C. scoparius* was measured as distance from the extracted soil core to the closest mature *C. scoparius* (determined by the presence of flowers or seedpods).

Response	Predictors (richness)	Effect size	*P*
Shoot mass (g)	All fungi	.	.
	Basidiomycota	.	.
	Glomeromycotina	0.40	**< 0.0001**
	Chytridiomycotina	.	.
	Plant pathogens	.	.
	Saproptrophs	.	.
	Distance from broom	.	.
%N (shoot)	All fungi	.	.
	Basidiomycota	.	.
	Glomeromycotina	.	.
	Chytridiomycotina	.	.
	Plant pathogens	-0.17	**0.0217**
	Saproptrophs	0.23	**0.0018**
	Distance from broom	.	.
%P (shoot)	All fungi	.	.
	Basidiomycota	.	.
	Glomeromycotina	.	.
	Chytridiomycotina	.	.
	Plant pathogens	-0.42	**0.0342**
	Saproptrophs	0.45	**0.0250**
	Distance from broom	.	.

Table 2. Linear mixed-effect model *P* value extimates and effect sizes for all species proportional abundance predictors and reponse variables which previously showed a significant response to *C. scoparius* at the plot-scale. Plant species and soil sampling plot were both set as random effects. "**.**" indicates term dropped during model simplification. Distance to *C. scoparius* was measured as distance from the extracted soil core to the closest mature *C. scoparius* (determined by the presence of flowers or seedpods). Prop. abund. = proportional abundance.

Response	Predictors (prop. abund.)	Effect size	*P*
Shoot mass (g)	Glomeromycotina	0.30	**< 0.0001**
	Chytridiomycotina	.	.
	Plant pathogens	.	.
	Distance from broom	-0.19	**0.0031**
%N (shoot)	Glomeromycotina	.	.
	Chytridiomycotina	.	.
	Plant pathogens	.	.
	Distance from broom	.	.
%P (shoot)	Glomeromycotina	.	.
	Chytridiomycotina	.	.
	Plant pathogens	.	.
	Distance from broom	.	.

Table 3. Linear mixed-effect model *P* value, *t* value and parameter estimates for shoot biomass, shoot % N and shoot % P. All predictors retained in the best model are reported, including terms marginal to significant interactions. All used explanatory variables are based of Tables 1 & 2. The explanatory variables for shoot biomass in the initial full model were AMF rarefied richness, AMF proportional abundance and distance to closest mature *C. scoparius* along with plant origin ('Native', TRUE/FALSE) and Fabaceae (TRUE/FALSE) status as fixed effects and with plant species and soil sampling plot as random effects. The explanatory variables for both shoot % N and shoot % P in the initial full model were plant pathogen rarefied richness and saprotroph rarefied richness along with plant origin and Fabaceae status as fixed effects (and plant species and soil sampling plot as random effects).

	Shoot biomass (g)		
	P	*t*	Parameter estimates
AMF richness	**< 0.0001**	0.269	0.025
Distance to closest mature broom	**0.0494**	-1.965	-0.131
Fabaceae	**0.0352**	3.394	1.063
Native	0.1860	0.966	0.302
AMF richness × Fabaceae	**< 0.0001**	7.284	0.771
AMF richness × Native	0.0643	1.825	0.191
Fabaceae × Native	**0.0072**	-2.684	-1.188
AMF richness × Fabaceae × Native	**< 0.0001**	-4.482	-0.669

	%N (shoot)		
	P	*t*	Parameter estimates
Saprotroph richness	**0.0342**	2.840	0.228
Fabaceae	**< 0.0001**	4.325	1.422
Native	0.1186	-0.472	-0.155
Saprotroph richness × Fabaceae	0.9408	-1.966	-0.216
Saprotroph richness × Native	0.5225	-2.388	-0.249
Fabaceae × Native	0.3671	-0.901	-0.420
Saprotroph richness × Fabaceae × Native	**0.0045**	2.841	0.439

	%P (shoot)		
	P	*t*	Parameter estimates
Plant pathogen richness	**0.0344**	-3.056	-0.716
Saprotroph richness	**0.0245**	3.063	0.716
Fabaceae	0.7336	-1.730	-0.534
Native	0.3764	-0.835	-0.256
Plant pathogen richness × Fabaceae	**0.0159**	2.411	0.623
Saprotroph richness × Fabaceae	**0.0272**	-2.209	-0.572
Fabaceae × Native	**0.0355**	2.102	0.921

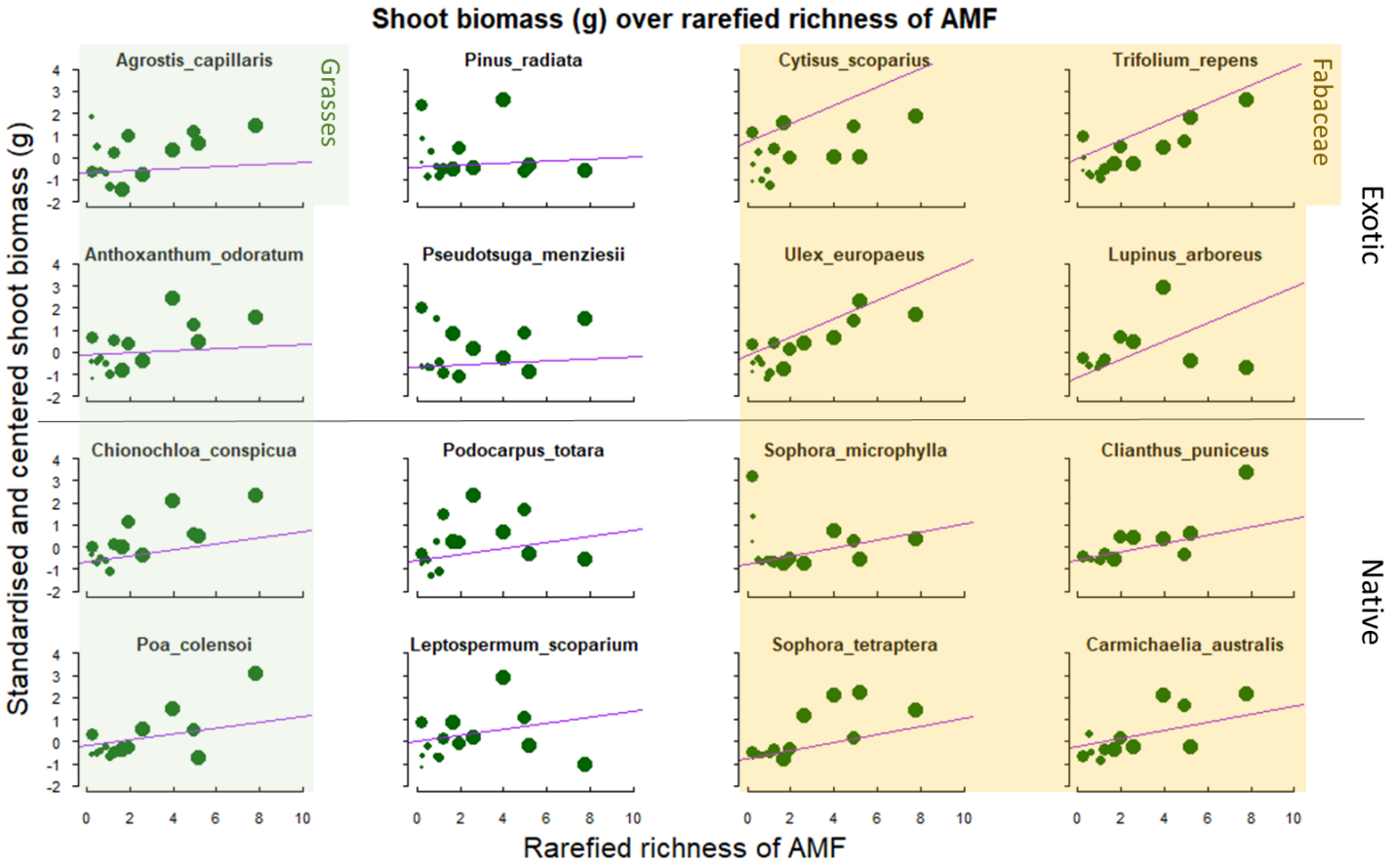


Figure 1. Standardised and centred aboveground dry biomass (g) over rarefied richness of AMF (i.e., Glomeromycotina). Size of points is scaled according to the square root of *C. scoparius* coverage. Lines were obtained from the created linear mixed-effect model via lme4's *predict* function.

Discussion

The results of this study suggest that increased AMF richness induced by the soil legacy of *C. scoparius* favours the growth of Fabaceae, particularly exotic Fabaceae, compared with non-N-fixing native plants. This finding matches the results of our field experiment, which likewise showed that the association of *C. scoparius* with soil microorganisms was most beneficial for exotic legumes compared to native legumes (Allen *et al.* (2020); Appendix E). Once AMF rarefied richness was included in my models, there was no residual effect of *C. scoparius* coverage on shoot biomass, inferring that AMF rarefied richness was sufficient to explain effects on shoot biomass. I accept my hypothesis that a change in fungal diversity induced by *C. scoparius* drives the mostly positive soil legacy effect of *C. scoparius* and can specify that AMF richness (as opposed to AMF proportional abundance) is here responsible.

Effect of AMF richness on plant growth

Although it was statistically supported that exotic Fabaceae benefited the most from increased AMF rarefied richness, this pattern was mostly attributed to *Trifolium repens* and *Ulex europaeus* and was least apparent for *Lupinus arboreus* (Figure 1). As *L. arboreus* is considered non-mycorrhizal (Oba *et al.* 2001), *L. arboreus* may to some extent serve as "the exception that proves the rule", considering that *L. arboreus* could not benefit from increased AMF richness due to not forming AMF associations.

A straightforward explanation for the increase in shoot biomass observed for Fabaceae would be that due to the lack of N-fixation by rhizobia, non-Fabaceae are more N-limited than Fabaceae and therefore cannot effectively profit from increased P acquisition from AMF as N remains a limiting factor. In more detail, I already know from the results of my greenhouse experiment that exotic Fabaceae in particular were putatively P limited (Koerselman and Meuleman 1996), whereas most non-Fabaceae were putatively N limited (Figure 6; Chapter 2). As such, having an increased P availability will not necessarily benefit the growth of N-limited plants (i.e., non-Fabaceae), whereas having an increased P availability is likely to benefit the growth of N-fixing plants which are not N-limited (i.e., Fabaceae). It is also worth noting that non-mycorrhizal *L. arboreus* was markedly more putatively P limited than other exotic Fabaceae (Figure 6; Chapter 2), which could be expected due to *L. arboreus* being unable to derive P from AMF (although see Wang *et al.* (2019) regarding the ability of some *Lupinus* sp. to instead form cluster roots to increase P uptake).

It is nonetheless uncertain as to why exotic Fabaceae benefit more from increased AMF richness than native Fabaceae. I know from the results of my natural survey (Chapter 3) that 1) *C. scoparius*

is possibly spreading alongside belowground mutualists, pathogens and commensals (Nuñez and Dickie 2014), while simultaneously forming cosmopolitan associations with widespread AMF communities across plots. I know from our field experiment that 2) the association of *C. scoparius* with soil microorganisms was most beneficial for exotic legumes compared to native legumes (Allen *et al.* (2020); Appendix E). Although the response of *C. scoparius* to AMF richness was not as strong as *Trifolium repens* or *Ulex europaeus*, considering points 1) and 2), it may be proposed that the exotic legumes in my experiment were more amenable to forming both novel and cosmopolitan AMF associations than native legumes and can therefore benefit more from available AMF symbioses. Although plant-AMF mutualisms are more promiscuous than plant-bacterial mutualisms (Klironomos *et al.* 2000), it has already been proposed that legumes native to New Zealand form different associations with N-fixing rhizobia compared with exotic *Cytisus scoparius* and *Ulex europaeus* (Weir *et al.* 2004). It may be the case that native and exotic Fabaceae likewise associate selectively with AMF, either in terms of more specific associations or an increased number of associations. Though not explicit to AMF, there is growing evidence that exotic plants integrate into broader, more generalist association networks (Stouffer *et al.* 2014, Rodríguez-Echeverría and Traveset 2015, Emer *et al.* 2016), although there are exceptions to this pattern as native grasses, for instance, can form more associations with a given diversity of AMF compared with non-native grasses (Jordan *et al.* 2012).

Different species of AMF have been known to lead to different degrees of plant P uptake (Miransari *et al.* 2009). The fact that AMF rarefied richness was a stronger predictor of plant biomass as opposed to AMF proportional abundance might indicate that a higher AMF richness correlates with an increased likelihood that one or several associating AMF confer relatively more P to plants. Plants can discriminate and reward the best AMF partners with more carbohydrates (Kiers *et al.* 2011). In turn, a plant's AMF can enforce cooperation by increasing nutrient transfer only to those roots providing more carbohydrates (Helgason *et al.* 2002, Kiers *et al.* 2011). A more speculative interpretation in a similar vein with Jansa *et al.* (2008) regarding plant growth and AMF richness would be that AMF are forced into inter-specific competition as a plant will preferentially associate itself with the species of AMF which supplies the greatest amount of benefits. A plant with a high diversity of AMF would receive P "at a bargain price", not only because the plant initially has a high number of AMF species which can supply P, but also because associating AMF species now need to compete against each other when "setting the price of nutrient exchange". Such belowground economics may however be biased toward plant growth in greenhouse experiments, as having a single plant per pot forces any existing AMF to rely on that particular plant for mutualism.

For a given plant species, soil obtained from closely related plants generally has a more negative effect on plant growth than soil obtained from distantly related plants (Kempel *et al.* 2018). Negative plant-soil feedbacks occur in part when pathogens accumulate in the rhizosphere of plant species (Kulmatiski *et al.* 2008). This accumulation of plant pathogens alongside *C. scoparius* coverage (both in terms of richness and proportional abundance) has been observed in my natural survey (Chapter 3). Regarding why *C. scoparius* did not show as notable an increase in shoot biomass over AMF richness compared to *T. repens* and *U. europaeus*, it is likely that negative feedback of plant pathogens more specific to *C. scoparius* counteracted the effect of increased AMF richness.

Experimental design considerations

Most experimental design considerations in this chapter overlap those in Chapters 2 & 3. Although I studied how soil chemical and soil biological attributes correlate with plant growth, the physical aspect of soil legacy has not been considered, despite having an important impact on plant development (Van der Putten *et al.* 2013). In the design of my greenhouse experiment, I mixed the soils I collected across a *C. scoparius* density gradient at a 1:1 volume ratio with washed river sand. This mixing was done to minimize the quantity of soil required (and thereby any disruption caused to the permanent sampling plots) as well as to help standardize soil porosity and generally reduce physical disparities between soils. Although obtained from the same 18 plots, I also cannot claim that the soil community to which plants were exposed to in my greenhouse experiment is the exact same soil community in my natural survey. However, as I examined changes in microbial composition across a *C. scoparius* density gradient (as opposed to a "Control and Effect" style experiment), I can be reasonably confident that the effects of both soil legacy and soil community composition overlap each other.

Conclusions and applications

Although soil legacy studies are increasingly popular (Klironomos 2003, Edwards *et al.* 2019, Wilschut *et al.* 2019), it is fairly rare that a soil legacy study traces growth responses back to fungal community composition. Though I previously thought that the removal of *C. scoparius* might benefit the growth of native plants due to the generally positive soil legacy of *C. scoparius*, I now see that AMF richness is integral to the legacy of *C. scoparius* and may benefit P-limited exotic Fabaceae to a greater extent than N-limited non-Fabaceae as well as native Fabaceae. I add that growth benefits to P-limited exotic Fabaceae are contingent on the ability of the exotic Fabaceae to form AMF associations, as non-mycorrhizal plants (i.e., *L. arboreus*) did not profit from an increase in AMF richness. A recent study has shown how plant growth may increase when a plant is co-inoculated with both AMF and certain bacteria (Bourles *et al.* 2020). A future research avenue would be to further examine how known differences in the rhizobial associations between native

and exotic legumes (Weir *et al.* 2004) might explain why exotic Fabaceae differ in their growth response to AMF richness compared to native Fabaceae.

Chapter 6: General discussion

Key findings

My PhD thesis represents a body of research work which combined a well-established soil legacy approach with more modern eDNA metabarcoding, enabling me to observe how a high diversity of fungi, particularly arbuscular mycorrhizal fungi, underlies the invasion success of *C. scoparius*. Although fungal diversity in soil under *C. scoparius* is higher than in grassland uninvaded by *C. scoparius, C. scoparius* invasion results in increased homogenisation of certain fungal groups within the overall soil fungal community. Increased arbuscular mycorrhizal richness, which is found in these more homogenised soil communities, is partly responsible for the generally positive soil legacy of *C. scoparius*, especially for exotic Fabaceae which can probably benefit more from AMF-facilitated P enrichment due to their ability to first fix required N for growth. The benefit that exotic Fabaceae derive from *C. scoparius* can be observed both in greenhouse studies as well as in the presence of live *C. scoparius*. I present the pitfalls and benefits of eDNA pooling, show a fungal taxon-wide bias in the proportional abundance of fungi in pooled samples, demonstrate how rarer fungi remain increasingly unaccounted for with increased degrees of pooling, yet also show how pooling may benefit researchers who wish to study larger-scale processes.

Considerations for eDNA community studies

There has generally been a correlation between the biodiversity of groups of directly or indirectly interacting organisms (Gaston 2000, Scherber *et al.* 2010, Peng *et al.* 2019). Having sampled from plots where *C. scoparius* formed near-monocultures, the most counterintuitive result in my thesis was the discovery that *C. scoparius* increased fungal species richness compared to uninvaded grassland. Soil biological responses to plant invasion do not evidently need to result in decreases in species richness as has been documented for native plants (D'Antonio and Flory 2017, Fahey *et al.* 2018), arthropods (Andersen *et al.* 2019, Jesse *et al.* 2020), birds (Grzędzicka and Reif 2020), small and large mammals (Ceradini and Chalfoun 2017, Dumalisile and Somers 2017), and amphibians (Nunes *et al.* 2019), among other taxa. The field of invasion ecology has generally held an aboveground focus (Dickie *et al.* 2017a), although more belowground studies have been conducted since the advent of eDNA metabarcoding. Knowledge of how fungal communities change across the density gradient of a plant invasion has not only permitted me to describe the consequences of a plant invasion, but also identify possible belowground enablers of plant invasion. Whereas the majority of invasion ecology studies focus on either the cause (e.g., habitat fragmentation) or effect (e.g., reduced richness) of a plant invasion, eDNA metabarcoding presents

a novel opportunity to consider both causes and effects simultaneously, while also obtaining a general overview of how species are distributed in certain sites.

Although eDNA metabarcoding has been implemented to assess New Zealand's terrestrial biodiversity (Holdaway *et al.* 2017), metabarcoding is subject to several biases (Bulman *et al.* 2018, Makiola *et al.* 2019b), which has led to calls for eDNA metabarcoding studies requiring robust experimental designs to draw sound ecological conclusions (Dickie *et al.* 2018, Zinger *et al.* 2019). I demonstrate in my eDNA pooling experiment (Chapter 4) that biases surrounding eDNA metabarcoding studies extend to eDNA sample pooling, which modifies both the observed rarefied richness and proportional abundance of fungi.

As analysing eDNA communities is a rapidly evolving and diverse field (Taberlet *et al.* 2018, Allwood *et al.* 2020), there are always variations in methodology to consider when undertaking eDNA studies. Although the soil extraction kit I used in my eDNA natural experiment (Chapter 3) and my eDNA pooling experiment (Chapter 4) was the same recommended by Lear *et al.* (2018), there still remains some uncertainty regarding the best available method to process soil samples (Hermans *et al.* 2018). It is known that eDNA metabarcoding studies are likewise affected by substrate selection (Koziol *et al.* 2019) and I can be reasonably confident that using deeper soil samples (deeper than 150-200 mm) would have impacted observed fungal communities (Schlatter *et al.* 2018, Sosa-Hernández *et al.* 2018). However, as exotic plants often have shallower roots than native species (Upton *et al.* 2020), studying the effect of *C. scoparius* on topsoil fungal communities does give a more reliable picture of changes in fungal communities induced by *C. scoparius*. Topsoil is also most relevant for understanding effects on seedlings of other species, as all seedlings start with their roots in the topsoil. A benefit of obtaining soil samples from permanent sampling plots laid out according to Hurst and Allen (1993) is that longer-term changes can be measured, which is relatively rare in soil community literature (although see Sielaff *et al.* (2018) or Song *et al.* (2020)).

As recommended by Dickie *et al.* (2018), I used both negative and positive controls in my experimental design to account for possible sample contamination (which proved low) and to set aside any DNA "naturally" found in extraction kits (Toole *et al.* 2019). As I performed my PCRs in duplicate for both my natural survey (Chapter 3) and my eDNA pooling experiment (Chapter 4), I can be more confident that my results are more reproducible (Bautista-de los Santos *et al.* 2016). A very important consideration for eDNA studies is the choice of primers. It was my initial intention to use the ITS7o primer, as it enables better identification of arbuscular mycorrhizal fungal communities (Kohout *et al.* 2014), which proved important throughout my results. However, an initial unsuccessful metabarcoded library submission with the ITS7o primer prompted me to revert to the more widely used ITS7 primer (Ihrmark *et al.* 2012). Other colleagues had reported similar issues, and it is probable that faulty DNA normalization and purification

plates had led to my initial unsuccessful eDNA library submission as opposed to an issue with the ITS7o primer itself. The clarity of my results would have nonetheless benefited from using the ITS7o primer instead of the ITS7 primer and it is probable that the diversity and proportional abundance estimates I obtained for arbuscular mycorrhizal fungi are underestimated.

It is unfortunately common that eDNA surveys give few details on the spatial arrangement and sampling design implemented (Dickie *et al.* 2018). Using a systematic method for sample collection (Hurst and Allen 1993), I was able to simultaneously 1) ensure the reproducibility of my results, 2) enable even comparisons between sampling plots, and 3) present my results via a measurement of soil community heterogeneity (i.e., true beta diversity (Whittaker 1970)), which is more easily understood by the public.

Lessons for soil legacy studies

The identity and composition of soil biota which may underlie plant growth responses or nutrient acquisition are rarely accounted for, even though soil microbiota can reduce plant growth through antagonism (Latz *et al.* 2016, Schroeder *et al.* 2020) and may determine plant nutrient availability (Edwards *et al.* 2019, Wilschut *et al.* 2019), among other effects. There have been calls to implement microbiome sequencing techniques in order to develop more predictive plant-soil feedback frameworks in certain ecosystems (Singh and Meyer 2020) and more generally understand how exotic plants can spread (Collins *et al.* 2019, Ramirez *et al.* 2019). My results in Chapter 5 show that knowing the fungal community inherent to the soil legacy of *C. scoparius* enabled a clearer understanding of the belowground processes which aid in the invasion of exotic Fabaceae, or more specifically, exotic Fabaceae which can form associations with AMF.

Soil legacy studies have moved from straightforward "Control and Effect" style experiments (Bever 1994) to more complex investigations which look at how both chemical and biological changes to soil composition induced by different densities of an invasive species alter the growth of plants with varying natural traits (Chapter 2; Chapter 5). Soil legacy studies incorporating soil communities are becoming increasingly popular (Detheridge *et al.* 2016, Pickett *et al.* 2019). Soil legacy responses can result in more dramatic changes to soil fungi compared to soil bacteria (Heinen *et al.* 2020), partly as fungi tend to be comparatively more spatially heterogeneously distributed in soil (Manter *et al.* 2010) (see however Collins *et al.* (2016) regarding how a plant can modify bacterial communities to a greater extent than fungal communities). Having studied fungal communities, a possible future research avenue would be to explore the communities of other soil micro-organisms, such as oomycetes (Cacciola and Gullino 2019), viruses (Sutela *et al.* 2019) and tardigrades (Bryndová *et al.* 2020), which may affect plant growth. Although every soil micro-organism can be an important determinant of soil legacy, considering which group or groups of soil micro-organisms could have impacted soil legacy studies could well increase our

understanding of plant-soil feedbacks. There have already been examples in the literature where both fungi and bacteria have been shown to be inherent to a plant's soil legacy (Bourles *et al.* 2020, Pan *et al.* 2020, Saia *et al.* 2020a), and it is likely that studying these soil communities will shed more light on belowground processes which contribute to the spread of exotic plants (Waller *et al.* 2020).

Considerations for the conservation of New Zealand's grassland

The rapid growth of *C. scoparius* along with the formation of seedbanks make *C. scoparius* extraordinarily resistant to eradication, even after applying herbicide (Tran *et al.* 2016, Haubensak *et al.* 2020). However, ecological restoration with native vegetation could suppress *C. scoparius,* which is shade intolerant (Watt *et al.* 2003b, Burrows *et al.* 2015). Plant-soil feedbacks are important when considering ecological restoration projects (Yelenik and Levine 2011, Wubs *et al.* 2016), as new generations of seedlings might benefit or suffer from changes imposed by a previous plant on its surrounding soil. Monitoring changes in fungal communities is likewise important in restoration projects (Yan *et al.* 2018) as there can be a strong link between fungal diversity and soil and plant properties (Tedersoo *et al.* 2016, Yang *et al.* 2017). An ecological restoration project involving the removal of *C. scoparius* is something of a double-edged sword both in terms of species richness and soil legacy.

In terms of species richness, removing near-monocultures of *C. scoparius* would obviously increase plant diversity at least in the short term, yet as I found a greater fungal richness in soil invaded by *C. scoparius* compared to uninvaded grassland with a higher plant species richness (Chapter 3)*, C. scoparius* removal may likely result in a decrease in fungal diversity integral to New Zealand's conservation efforts (Holdaway *et al.* 2017, de Lange *et al.* 2018, Dickie *et al.* 2020). Moreover, as I unexpectedly found a greater proportion of rare fungi in plots with high *C. scoparius* coverage, removing large *C. scoparius* invasions might possibly endanger rarer local soil-dwelling fungi (although see Dickie *et al.* (2020) concerning how rare wood inhabiting fungi are not local). However, it is important to consider the relatively small scale at which I undertook my natural survey. Although the increased productivity induced by *C. scoparius* may putatively enable more fungal OTUs to exist near *C. scoparius* (Chapter 3), I also know that *C. scoparius* decreases heterogeneity of some fungal groups, and it is probable that broader scale eDNA sampling of grassland uninvaded by *C. scoparius* will reveal a greater fungal diversity compared to the more homogeneous fungal communities near *C. scoparius*.

In terms of soil legacy, soil obtained after removing live *C. scoparius* does increase overall plant growth in a greenhouse environment (Chapter 2), although exotic Fabaceae benefited more than native Fabaceae in both my greenhouse experiment and our field experiment (Allen *et al.* (2020); Appendix E). *Cytisus scoparius* frequently grows in New Zealand's forestry plantations where it is

considered detrimental to the country's pine industry, particularly for *Pinus radiata* (Tran *et al.* 2016). Although generally regarded as a serious issue in early stages of pine development (Carter *et al.* 2019a), it is possible that *C. scoparius* might actually benefit older pine stands partly through N enrichment. This benefit is apparent for young *P. radiata* in a greenhouse environment, which show increased shoot % N and increased shoot N:P ratio over *C. scoparius* coverage (Chapter 2). My results are, however, based on soil free from live *C. scoparius*, which may be difficult to achieve in the field once *C. scoparius* is established (Haubensak *et al.* 2020). One innovative way to restore ecosystems following an invasion by non-native Fabaceae has been to allow the Fabaceae to "run its course", which then permits native flora to benefit from the Fabaceae's N-fixation, as has been implemented in Hinewai Reserve (New Zealand) for *Ulex europaeus* (Wilson *et al.* 2017). *Cytisus scoparius* can in some sites be regarded as a nurse crop for recovery of indigenous woody vegetation (Burrows *et al.* 2015). It can often be only a matter of time before plant pathogen-induced plant-soil feedback restricts the growth of older *C. scoparius* invasions, allowing other plants to benefit from increased soil N availability (Burrows *et al.* 2015).

More broadly in terms of plant invasions, prevention is the best cure (Hulme 2020). Five biocontrol agents have already been introduced to New Zealand to combat the spread of *C. scoparius* (Syrett *et al.* 1999, Syrett *et al.* 2007, Paynter *et al.* 2012), yet the shrub has become so commonplace in New Zealand that it now commonly regarded as part of the country's 'natural' landscape. For example, *C. scoparius* can be seen in promotional pictures for New Zealand's tourism industry (www.tourismnewzealand.com; accessed 16/06/2020). Plants from the taxonomic family of *C. scoparius* (Fabaceae) already have a long history of being invasive in areas which have undergone disturbance (Figueiral and Bettencourt 2004). Reinhart *et al.* (2017) observed that plant invasiveness is generally not associated with mycorrhizal responsiveness, yet acknowledge that mycorrhizal responsiveness can contribute to invasiveness in certain species. *Cytisus scoparius* and other leguminous shrubs (e.g., *Ulex europaeus*) may be the exception rather than the norm in terms of using AMF as a means to competitively increase growth. Considering the dependence of exotic Fabaceae on belowground symbionts, there is a silver lining in that plants can take several years to cultivate typical microbial communities and it likewise takes several years before plant-soil feedbacks come into full effect (Kulmatiski and Beard 2011). Now having a clearer image of the underlying mechanisms of *C. scoparius*, conservation effort should be prioritised toward restricting the spread of exotic Fabaceae which exist in relatively smaller ranges of New Zealand, before the invasion of these Fabaceae gains too much momentum. A list of such exotic Fabaceae along with their distribution throughout New Zealand can be found in Howell and Terry (2016) and include *Dipogon lignosus* and *Genista monspessulana.*

Future research interest: Generalist interactions between C. scoparius and belowground symbionts

Plants can share specific fungal symbionts at the level of populations or species (Dickie *et al.* 2017b). In a similar way as interaction networks have been applied, for example, in pollination biology to study which plants interact with which insects (Traveset *et al.* 2013), interaction networks may also be applied to study close associations between fungi and plants (Dickie *et al.* 2017a, Zenni *et al.* 2017, Xiao *et al.* 2018). Although some plants have been shown to be successful invaders via a single fungal symbiont (Hayward *et al.* 2015), having multiple fungal symbionts might also account for a plant's improved growth (Higo *et al.* 2018, Crossay *et al.* 2020). Having analysed soil samples as opposed to root samples with associating fungi, I cannot be certain that the increased richness of AMF I found in soil with higher *C. scoparius* density interacts with *C. scoparius*. Nonetheless, my results do indicate that apart from *C. scoparius* increasing the richness of surrounding fungi (including AMF), *C. scoparius* may putatively form more interactions with more species of AMF compared to other plants in uninvaded grasslands (Chapter 3).

A future research interest would be to analyse *C. scoparius* and its belowground symbionts via interaction networks. For each soil sample I used in my natural survey, I have also collected accompanying root samples with associating fungi, which may yield the raw data necessary for network analysis. Given the increases in soil fungal richness linked with higher densities of *C. scoparius*, I would hypothesize that *C. scoparius* is generalist in its associations with belowground mutualists to the extent that *C. scoparius* may act as a figurative "fungal sponge". Alien tree-ectomycorrhizal communities have been shown to be able to form their own network in novel ranges as well as form new linkages with native interaction networks (Dickie *et al.* 2017b), which may underlie their invasiveness. I am keen to discover whether non-native legume-arbuscular mycorrhizal networks likewise integrate into native networks.

Parting words

Metabarcoding is an exciting technique which can bring fresh insight to long-studied ecological processes such as invasion (Deiner *et al.* 2017) and plant-soil feedback (Dierks *et al.* 2019). Whereas invasion ecology has classically held an aboveground focus, much remains to be said regarding microbial invasions and plants co-invading with belowground mutualists (Dickie *et al.* 2017a). Sequencing eDNA has allowed us to gain insight into mostly hidden belowground communities, however this process comes hand in hand with challenges in terms of how to set-up experiments and interpret obtained results (Dickie *et al.* 2018, Lear *et al.* 2018). Two key themes which underlie my results are the importance of regulation and integration. Regulation of metabarcoding studies is required to accurately draw out reproducible conclusions and integrating soil legacy studies with eDNA data can provide a more comprehensive view of processes underlying plant invasion.

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Appendix A (Supplement Chapter 2)

A1. Coordinates of 18 marked permanent sampling plots in Molesworth used for soil collection. All plots are within 2.5 km of each other at an altitude 872-933 m above sea level. The plots were set up by Manaaki Whenua and follow field protocols outlined by Hurst and Allen (1993).

Plot	East Longitude	North Latitude
MW1	24°96'108"	58°60'748"
MW2	24°96'131"	58°60'767"
MW3	24°96'171"	58°60'777"
MW6	24°96'121"	58°60'592"
MW7	24°96'095"	58°60'563"
MW12	24°96'348"	58°60'808"
MW13	24°96'011"	58°60'642"
MW14	24°96'051"	58°60'651"
MW15	24°95'971"	58°60'651"
MW17	24°95'592"	58°60'764"
MW18	24°95'673"	58°60'782"
MW19	24°95'651"	58°60'673"
MW20	24°95'630"	58°60'779"
MW23	24°95'753"	58°60'794"
MW24	24°95'769"	58°60'824"
MW25	24°95'788"	58°60'780"
MW26	24°95'822"	58°60'755"
MW29	24°95'855"	58°60'681"

A2. *[Corresponds to Table 3]* Linear mixed-effect model *t*-values for *C. scoparius* coverage × plant origin × legume status (top table) and *C. scoparius* coverage × plant origin × ectomycorrhizal (ECM) status (bottom table). Non-significant terms only included if part of significant higher level interaction. "**.**" indicates term dropped during model simplification.

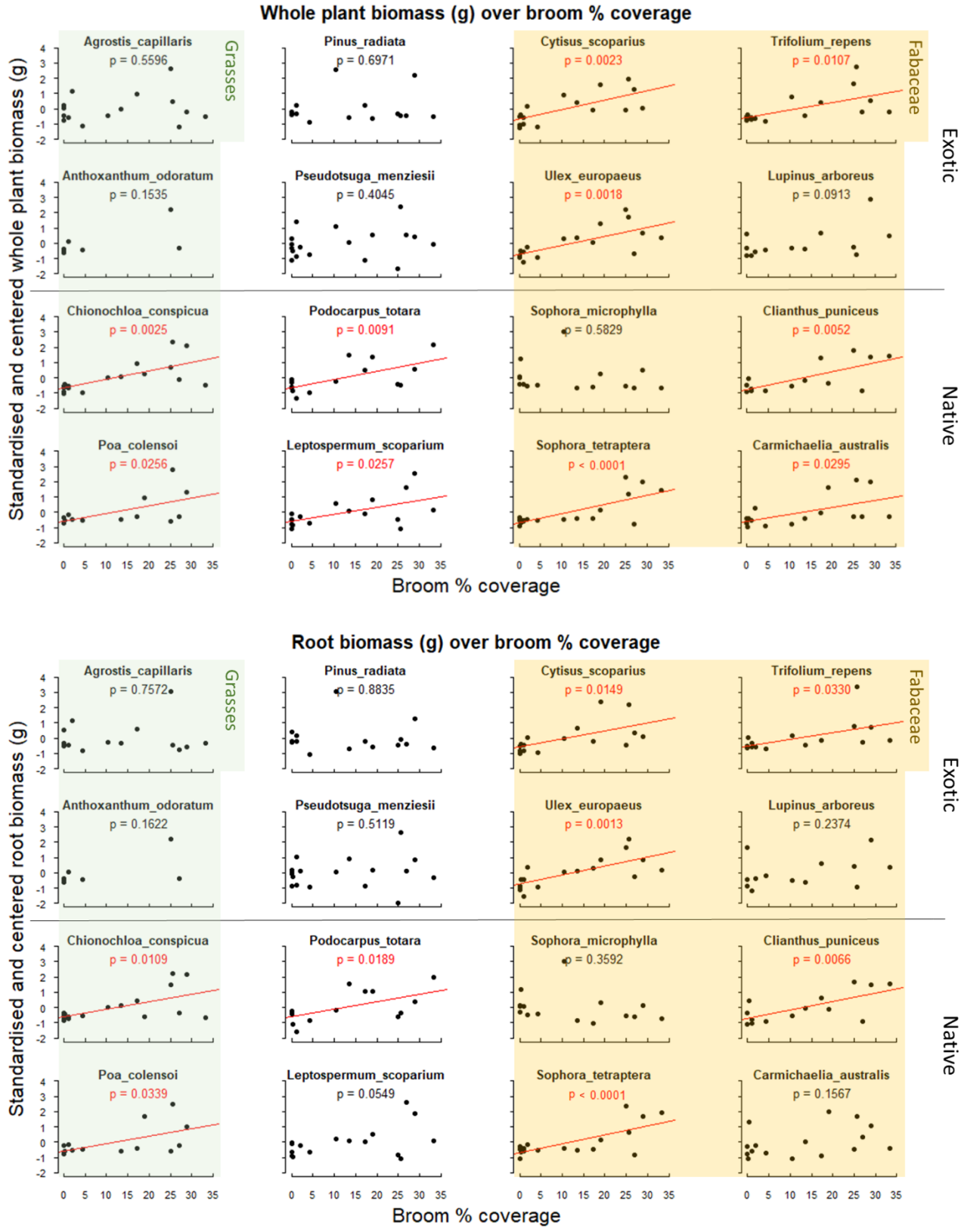
	Broom coverage	Fabaceae	Native	Broom coverage × Fabaceae	Broom coverage × Native	Fabaceae × Native	Broom coverage × Fabaceae × Native
Shoot mass (g)	1.024	1.070	0.228	6.629	2.112	-0.919	-5.014
Root mass (g)	0.267	-0.282	0.474	3.848	2.006	-0.464	-3.158
Whole plant mass (g)	0.308	0.992	0.485	5.862	1.699	-1.039	-3.992
Shoot N (%)	2.461	4.875	0.058	-2.402	-1.709	-1.643	2.464
Shoot P (%)	.	-1.743	-0.850	.	.	2.119	.
Shoot N (%) / P (%)	1.986	3.989	0.215	.	-3.320	-1.962	.
Total N / Total P	0.746	1.749	0.148	7.241	0.924	-1.105	-4.907

	Broom coverage	ECM	Native	Broom coverage × ECM	Broom coverage × Native	ECM × Native	Broom coverage × ECM × Native
Shoot mass (g)	6.506	-0.633	-0.901	-4.282	-3.376	0.918	3.275
Root mass (g)	4.700	0.345	-0.655	-2.438	.	1.989	.
Whole plant mass (g)	6.717	-0.233	-0.470	-4.433	-3.538	0.966	2.281
Shoot N (%)	0.425	-2.756	.	2.680	.	.	.
Shoot P (%)	.	.	.	.	.	.	.
Shoot N (%) / P (%)	0.740	-1.635	-1.407	3.544	-2.769	.	.
Total N / Total P	7.057	-0.862	-1.033	-4.031	-4.331	0.664	2.848

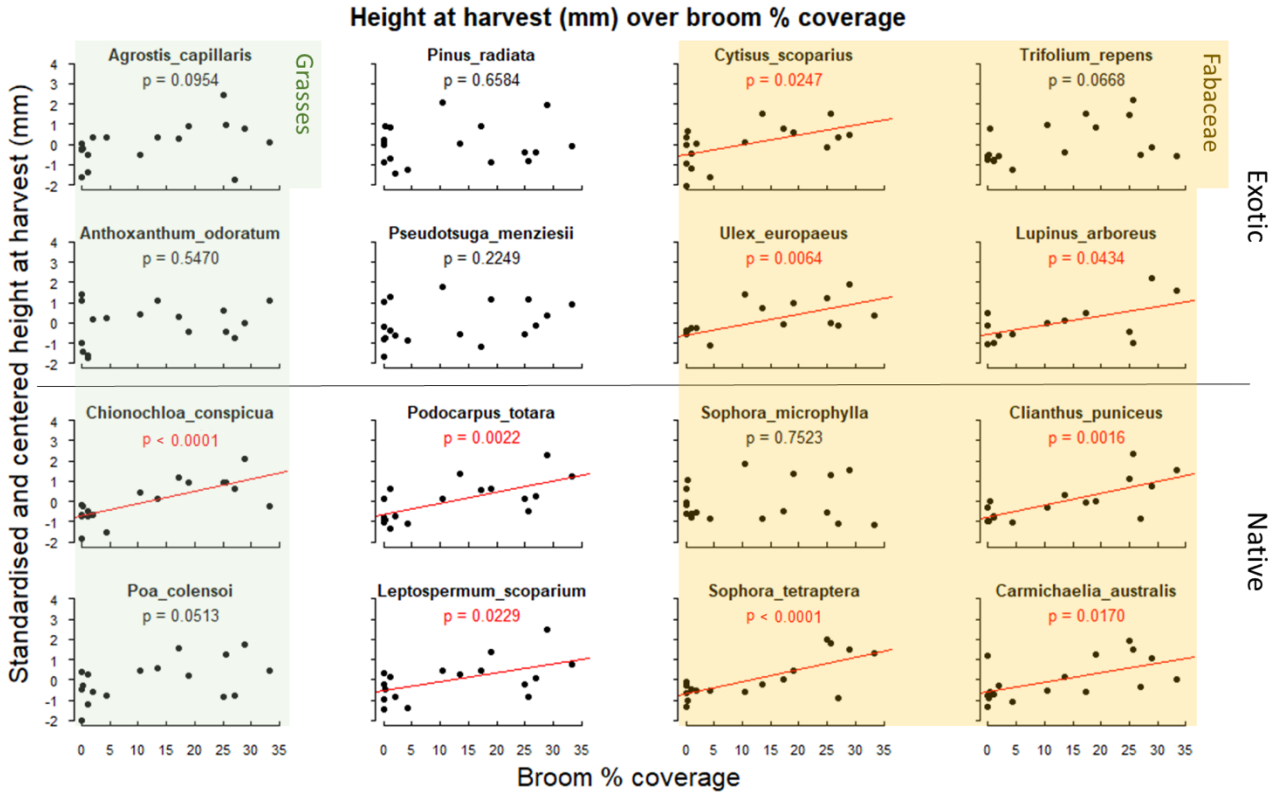
A3. *[Corresponds to Table 3]* Linear mixed-effect model estimates for log-transformed distance to closest mature *C. scoparius* × plant origin × legume status. *t*-values are outside of parentheses, *P*-values within parentheses. Non-significant terms only included if part of significant higher level interaction. "**.**" indicates term dropped during model simplification. Underlined values show which estimates differ in significance between the estimates in Table 3.

	Distance to broom	Fabaceae	Native	Distance to broom × Fabaceae	Distance to broom × Native	Fabaceae × Native	Distance to broom × Fabaceae × Native
Shoot mass (g)	**-0.888 (< 0.0001)**	**7.441 (0.0339)**	2.113 (0.1774)	**-6.622 (0.0003)**	**-1.884 (0.0201)**	**-5.797 (0.0068)**	**5.127 (< 0.0001)**
Root mass (g)	**-0.329 (< 0.0001)**	4.367 (0.9352)	2.516 (0.9739)	-4.122 (0.0525)	-2.155 (0.3774)	-4.230 (0.0549)	**3.795 (0.0012)**
Whole plant mass (g)	**-0.281 (< 0.0001)**	**7.094 (0.0349)**	2.131 (0.2761)	**-6.275 (< 0.0001)**	**-1.818 (0.0190)**	**-5.378 (0.0084)**	**4.711 (< 0.0001)**
Shoot N (%)	-2.143 (0.1594)	**0.222 (< 0.0001)**	-1.591 (0.1179)	2.027 (0.5506)	1.547 (0.9824)	1.640 (0.3720)	**-2.305 (0.0211)**
Shoot P (%)	.	-1.743 (0.7259)	-0.850 (0.3789)	.	.	**2.119 (0.0341)**	.
Shoot N (%) / P (%)	-2.218 (0.4828)	**3.310 (0.0009)**	**-4.046 (0.0155)**	.	**3.258 (0.0011)**	.	.
Total N / Total P	**-0.647 (< 0.0001)**	**8.479 (0.0005)**	**0.926 (0.0156)**	**-7.187 (< 0.0001)**	**-0.790 (0.0007)**	**-5.873 (0.0017)**	**5.025 (< 0.0001)**

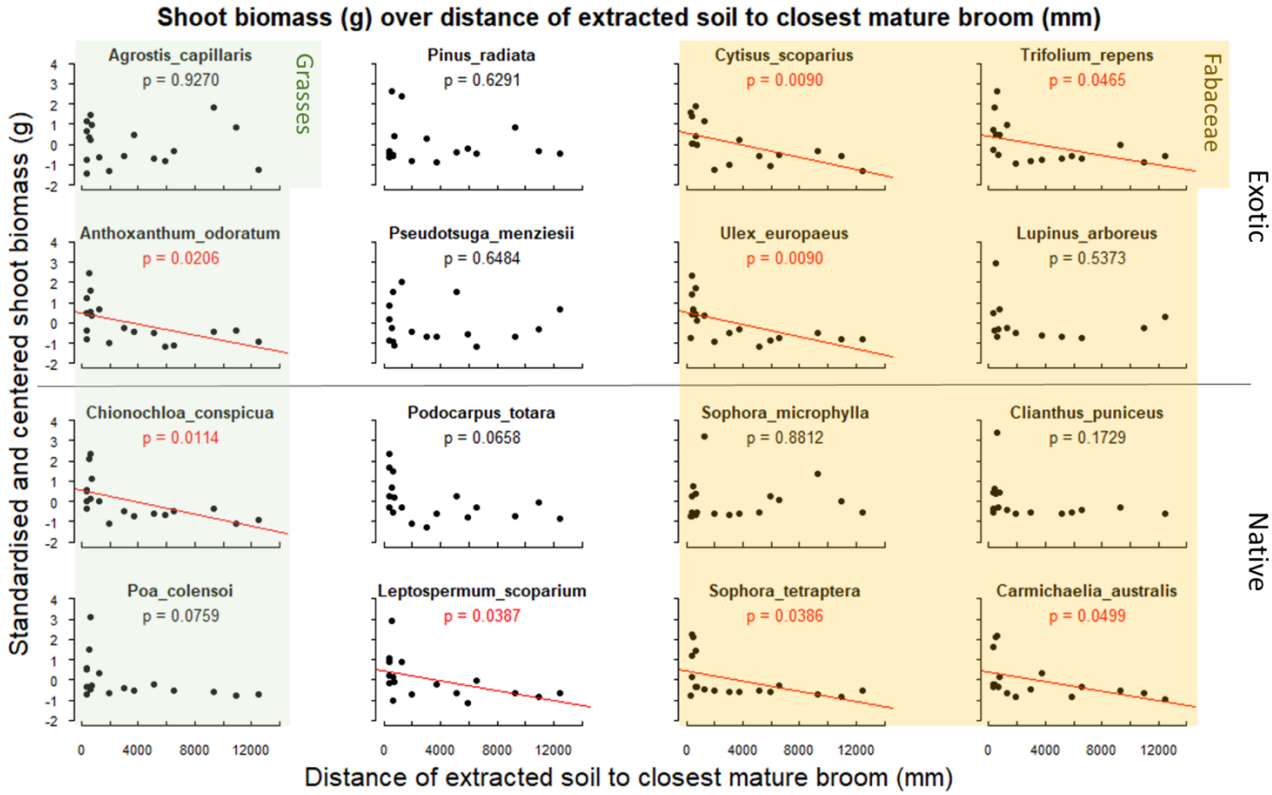
A4. *[Corresponds to Figure 4]* Standardised and centered root biomass, whole plant biomass and height at harvest for each plant over *C. scoparius* % coverage. Regression lines are shown when $P < 0.05$.



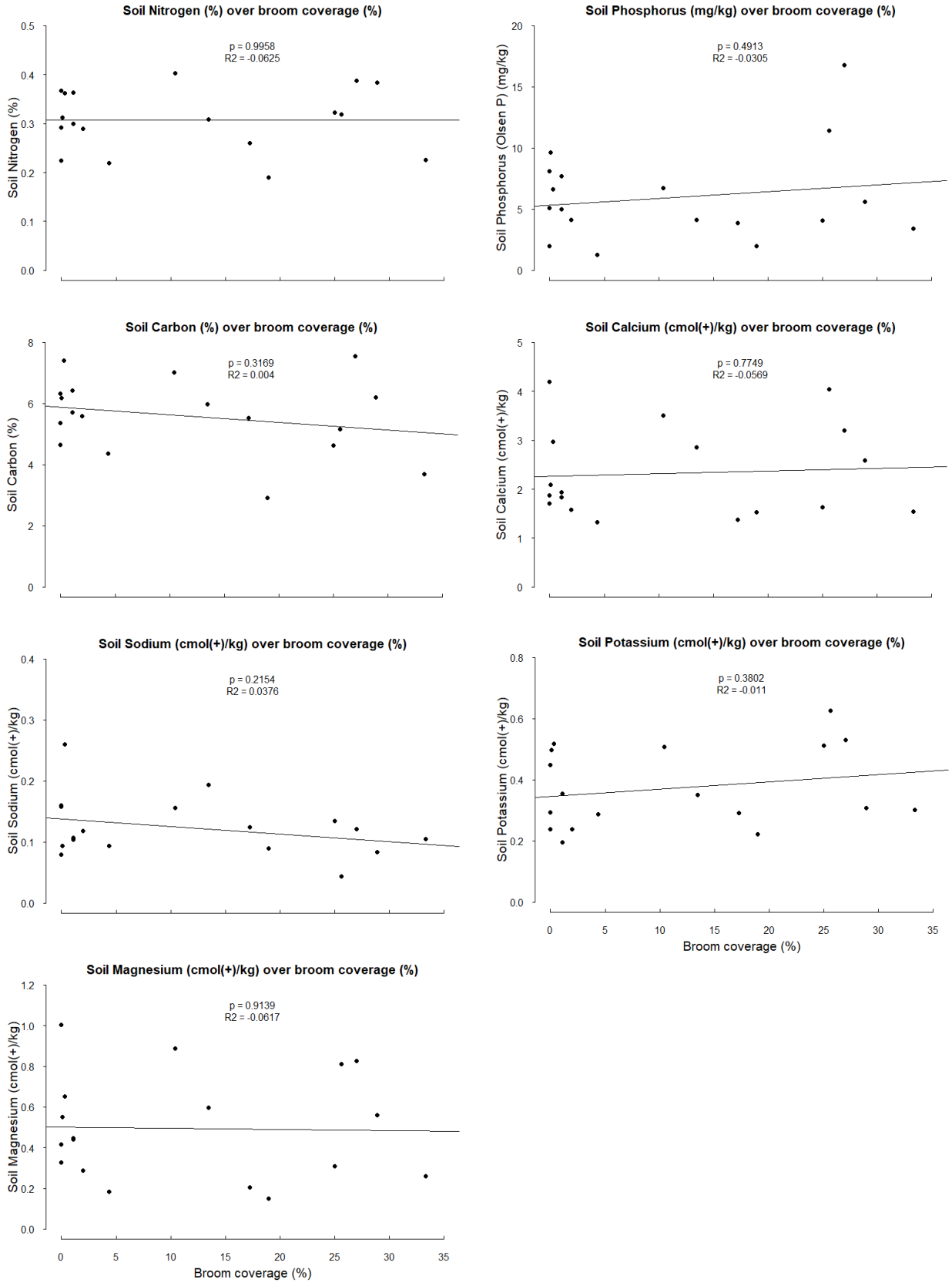
A4. *[Continued]*



A5. *[Corresponds to Figure 4]* Standardised and centered aboveground biomass for each plant over distance to the closest mature *C. scoparius*. Regression lines are shown when $P < 0.05$.

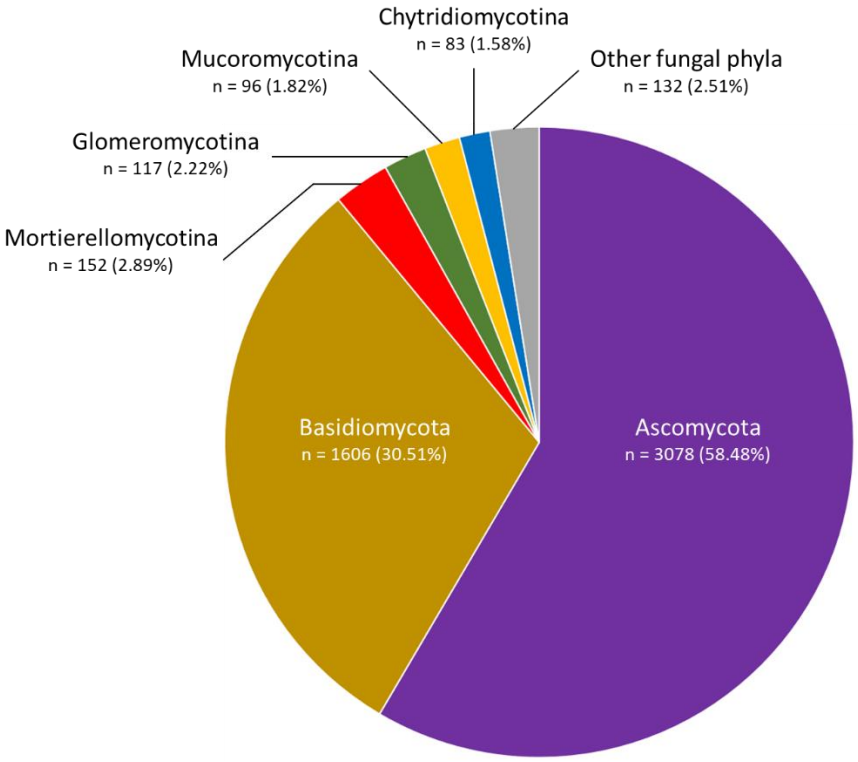


A6. Soil nutrients over *C. scoparius* % coverage. *P* values and adjusted R^2 are given in the plots.



Appendix B (Supplement Chapter 3)

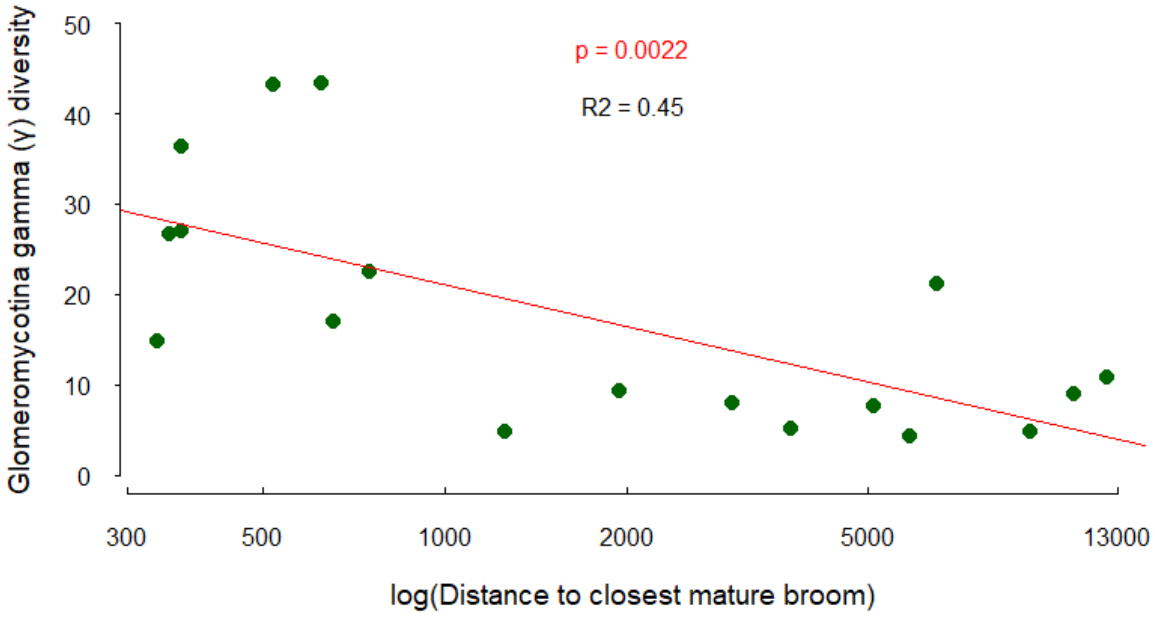
B1. Pie chart of 5263 fungal OTUs according to fungal taxa. The most abundant member of Ascomycota, Basidiomycota, Mortierellomycotina, Glomeromycotina, Mucoromycotina and Chytridiomycotina were a species of *Lecanicillium, Serendipita, Mortierella, Archaeosporaceae* (within the family of Glomales), *Umbelopsis* and *Phlyctochytrium* respectively.

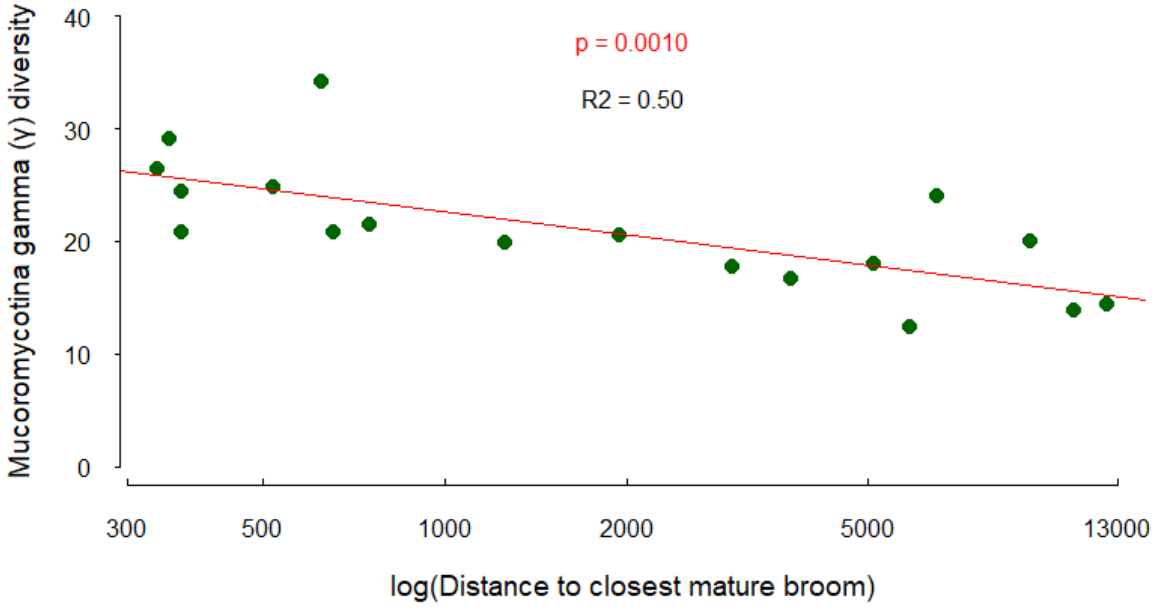


B2. Gamma (γ) diversity of fungal OTUs according to fungal taxa and plot. Plots are in ascending order of mean *C. scoparius* coverage and the first three plots (MW13, MW19 and MW26) contain no *C. scoparius*. Note that gamma diversity is not presented as integers because the mean rarefied gamma diversity was calculated across 250 iterations.

Plot	Asco-mycota	Basidio-mycota	Glomero-mycotina	Mortierello-mycotina	Chytridio-mycotina	Mucoro-mycotina	Other fungal taxa	**γ diversity per plot**
MW13	763.1	270.4	10.8	39.1	7.2	14.4	19.2	**1124.1**
MW19	946.7	403.3	21.1	43.9	7.9	23.9	13.8	**1460.7**
MW26	648.8	293.2	9.0	58.8	8.7	13.8	13.6	**1045.8**
MW7	580.7	255.9	4.3	49.6	1.0	12.4	6.3	**910.2**
MW6	588.2	258.1	4.8	50.8	3.3	20.0	17.4	**942.6**
MW1	641.3	265.0	8.0	47.2	5.3	17.7	16.1	**1000.6**
MW23	618.7	243.6	7.6	48.1	5.1	18.0	9.2	**950.3**
MW18	512.7	171.5	5.2	40.0	3.7	16.7	9.9	**759.7**
MW15	753.5	279.7	9.3	34.3	13.6	20.6	23.6	**1134.6**
MW12	636.4	284.7	4.8	51.3	5.2	19.8	11.3	**1013.4**
MW3	926.4	348.4	16.9	53.9	15.6	20.7	28.9	**1410.8**
MW14	701.1	271.7	22.5	48.6	9.7	21.5	16.9	**1092.1**
MW2	605.0	325.1	36.3	42.0	10.6	20.8	15.4	**1055.3**
MW25	484.4	269.6	26.9	49.4	5.6	24.4	11.0	**871.3**
MW17	754.8	328.5	43.4	40.5	8.0	34.1	19.8	**1229.2**
MW20	714.9	305.4	14.7	50.1	5.3	26.4	13.5	**1130.4**
MW29	800.8	376.5	43.1	51.4	7.9	24.8	13.4	**1317.8**
MW24	676.0	298.3	26.7	49.7	10.4	29.1	21.3	**1111.6**

B3. *[Corresponds to Figure 3]* Gamma (γ) diversity of Glomeromycotina (above) and Mucoromycotina (below) over distance from the extracted soil core to the closest mature *C. scoparius* (mm) (note log-transformed axis). *P* and R^2 values are given in the plots.





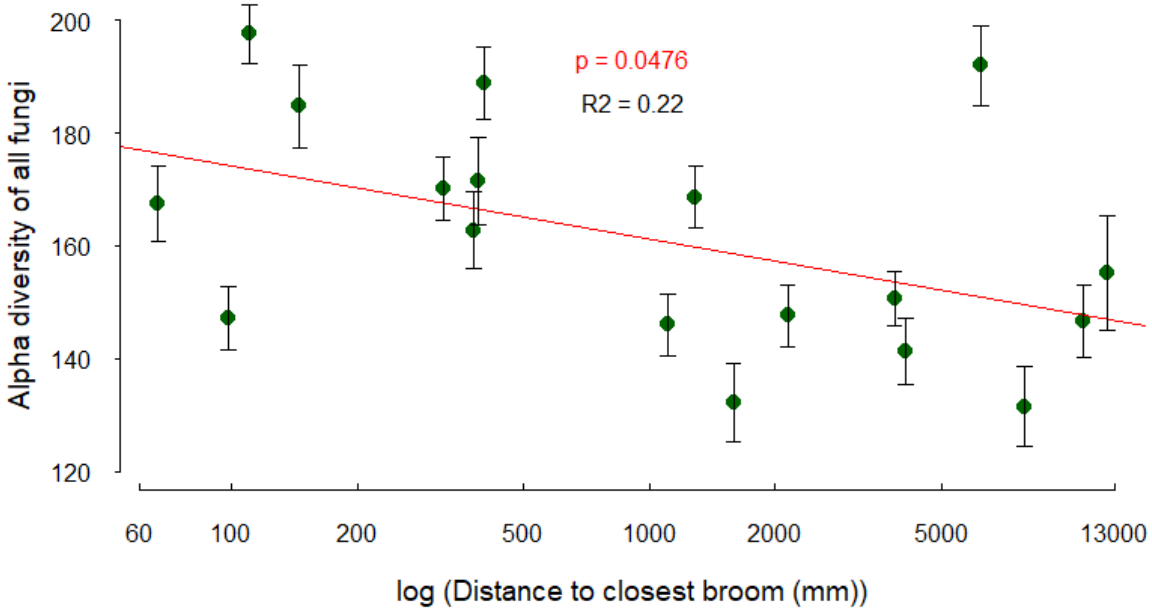
B4. *[Corresponds to Table 1]* Linear mixed-effect model *t* value estimates for alpha diversity (at the level of soil cores) and different measurements of *C. scoparius* density, i.e., *C. scoparius* % coverage and log-transformed distance to closest *C. scoparius* (mm). *t* value estimates are likewise given for proportional abundance and different measurements of *C. scoparius* density, except for all fungi (indicated by "**.**").

	Alpha Diversity		Proportional Abundance	
	Broom % Coverage	log(Distance to Broom)	Broom % Coverage	log(Distance to Broom)
All fungi	2.289	-3.025	.	.
Ascomycota	0.587	-2.335	-2.871	0.714
Basidiomycota	3.917	-3.211	1.544	-1.210
Glomeromycotina	1.495	0.065	-1.349	0.554
Mortierellomycotina	3.432	0.661	2.638	0.631
Chytridiomycotina	3.880	-2.919	1.505	-2.814
Mucoromycotina	0.690	-0.589	1.260	-0.326
Antagonists	3.043	-1.901	0.936	0.788
Symbiotrophs	0.103	-1.859	-2.157	0.551
Saprotrophs	1.992	-2.867	0.962	-0.465
Plant pathogens	3.014	-1.711	2.767	-3.094
Pathogens of fungi	-0.899	-0.682	1.691	-0.303
ECM (FUNGuild)	-0.353	-1.492	-0.795	0.040

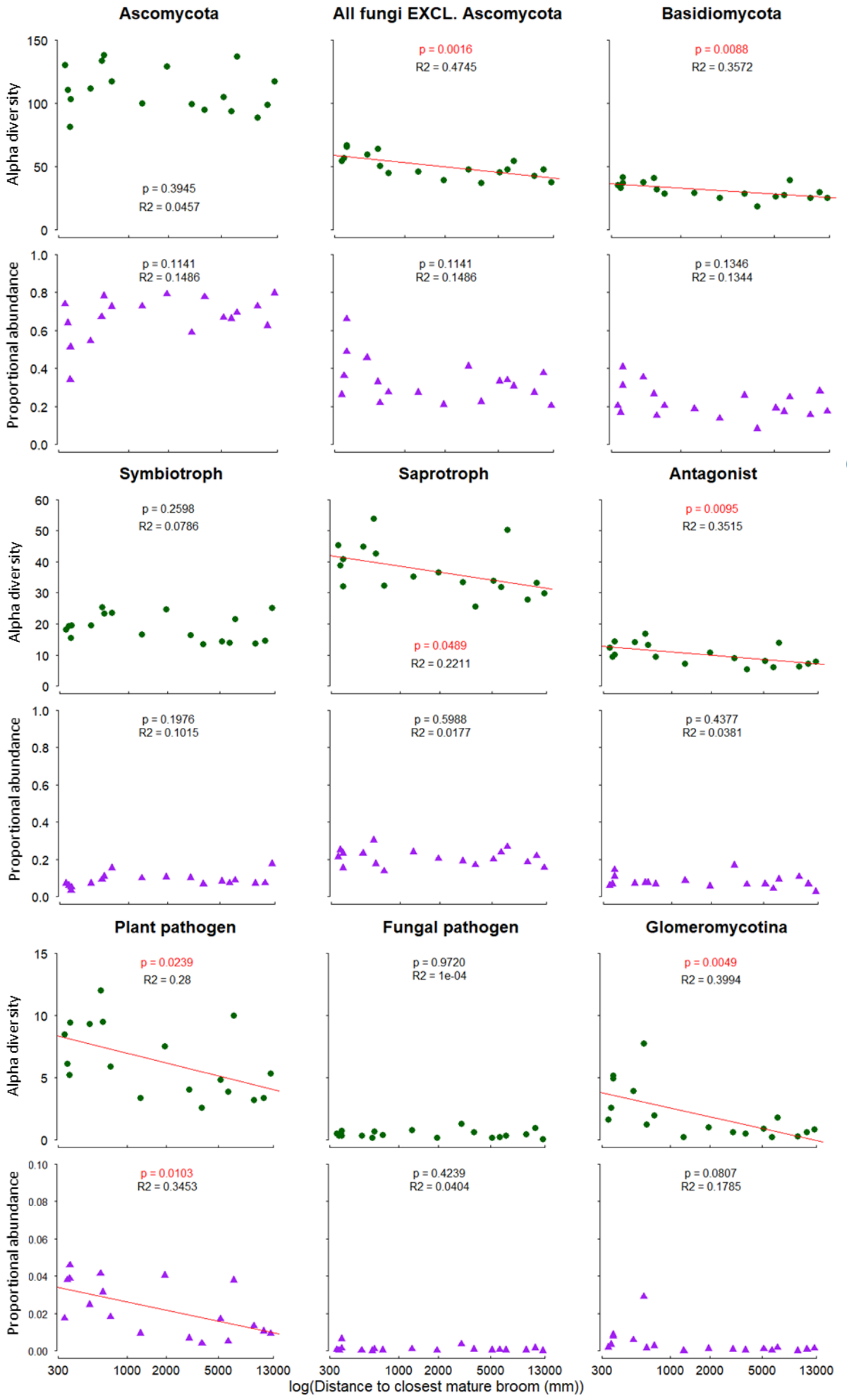
B5. *[Corresponds to Table 1]* Linear mixed-effect model estimates for alpha diversity (at the level of soil cores) and proportional abundance and different measurements of *C. scoparius* density, i.e., *C. scoparius* % coverage and log-transformed distance to closest *C. scoparius* (mm). *P* values are given within parenthesis, *t* value outside of parentheses.

	Alpha Diversity		Proportional Abundance	
	Broom % Coverage	log(Distance to Broom)	Broom % Coverage	log(Distance to Broom)
NOT Ascomycota	4.662 **(< 0.0001)**	-2.825 **(0.0047)**	2.871 **(0.0040)**	-0.714 (0.4754)
NOT Basidiomycota	1.431 (0.1523)	-2.547 **(0.0108)**	-1.544 (0.1227)	1.210 (0.2262)
NOT Glomeromycotina	2.161 **(0.0306)**	-2.938 **(0.0033)**	1.349 (0.1773)	-0.554 (0.5797)
NOT Mortierellomycotina	1.810 (0.0702)	-2.952 **(0.0031)**	-2.638 **(0.0083)**	-0.631 (0.5281)
NOT Chytridiomycotina	2.233 **(0.0255)**	-2.990 **(0.0027)**	-1.505 (0.1324)	2.814 **(0.0048)**
NOT Mucoromycotina	2.233 **(0.0255)**	-3.004 **(0.0026)**	-1.505 (0.1324)	2.814 **(0.0048)**
Antagonists (Loose)	2.505 **(0.0122)**	-3.455 **(0.0005)**	2.893 **(0.0038)**	-1.269 (0.2046)
Symbiotrophs (Loose)	1.963 **(0.0496)**	-2.284 **(0.0223)**	-1.371 (0.1704)	0.444 (0.6569)
Saprotrophs (Loose)	2.391 **(0.0168)**	-2.902 **(0.0037)**	0.413 (0.6799)	-0.153 (0.8786)
NOT Antagonists (Strict)	2.125 **(0.0336)**	-3.006 **(0.0026)**	-0.936 (0.3491)	-0.788 (0.4306)
NOT Symbiotrophs (Strict)	2.623 **(0.0087)**	-3.102 **(0.0019)**	2.157 **(0.0309)**	-0.551 (0.5817)
NOT Saprotrophs (Strict)	2.224 **(0.0261)**	-2.831 **(0.0046)**	-0.962 (0.3360)	0.465 (0.6416)
NOT Antagonists (Loose)	1.955 (0.0506)	-2.362 **(0.0182)**	-2.893 **(0.0038)**	1.269 (0.2046)
NOT Symbiotrophs (Loose)	2.418 **(0.0156)**	-3.133 **(0.0017)**	1.371 (0.1704)	-0.444 (0.6569)
NOT Saprotrophs (Loose)	1.557 (0.1194)	-2.518 **(0.0118)**	-0.413 (0.6799)	0.153 (0.8786)
ECM (FUNGuild) (Loose)	-1.089 (0.2763)	0.271 (0.7867)	-2.326 **(0.0200)**	1.163 (0.2446)

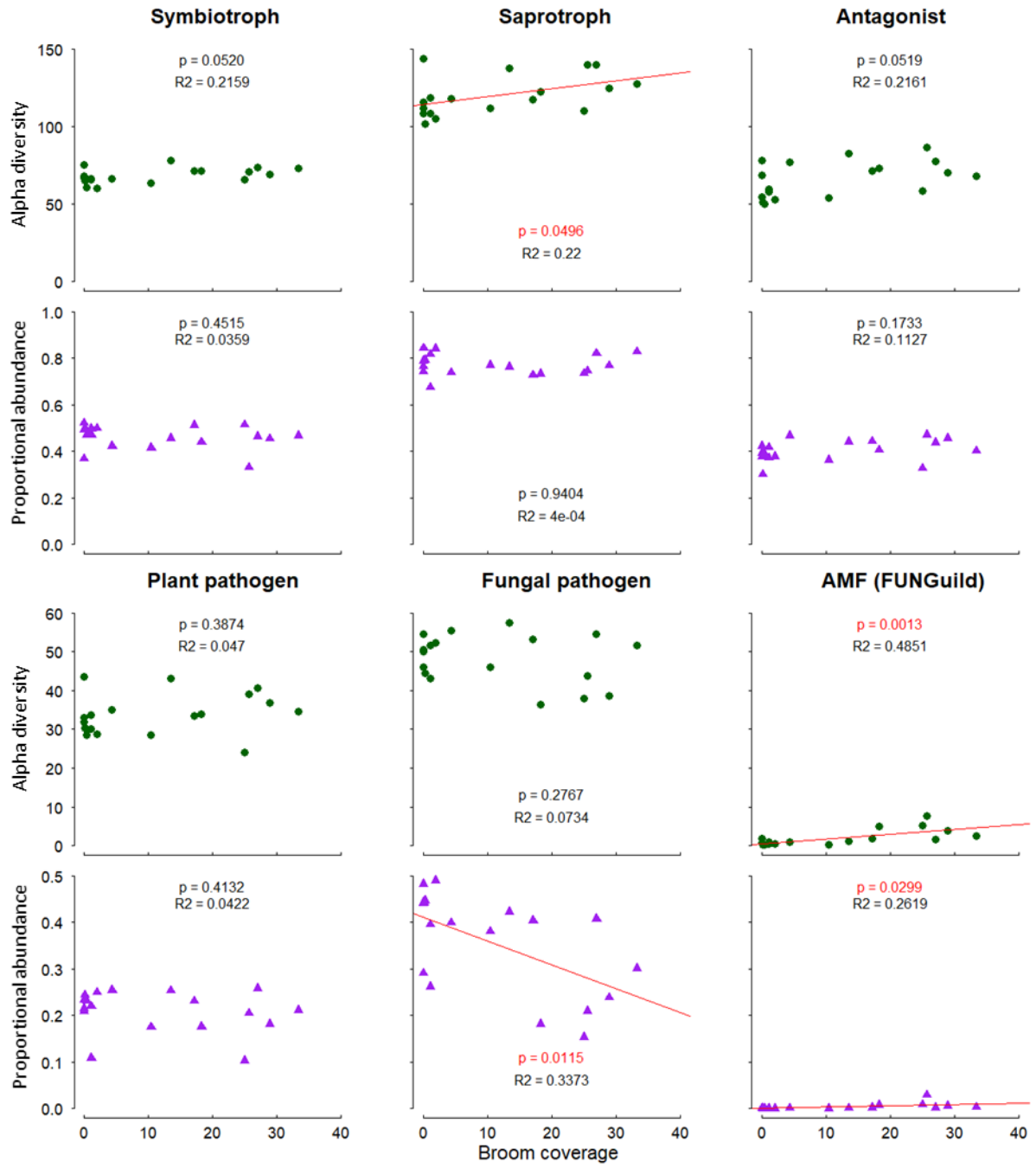
B6. *[Corresponds to Figure 4]* Mean alpha diversity per plot of all fungal OTUs over log-transformed distance from the extracted soil core to the stem of the closest *C. scoparius* (whether mature or immature). *P* and R^2 values are given in the plots.



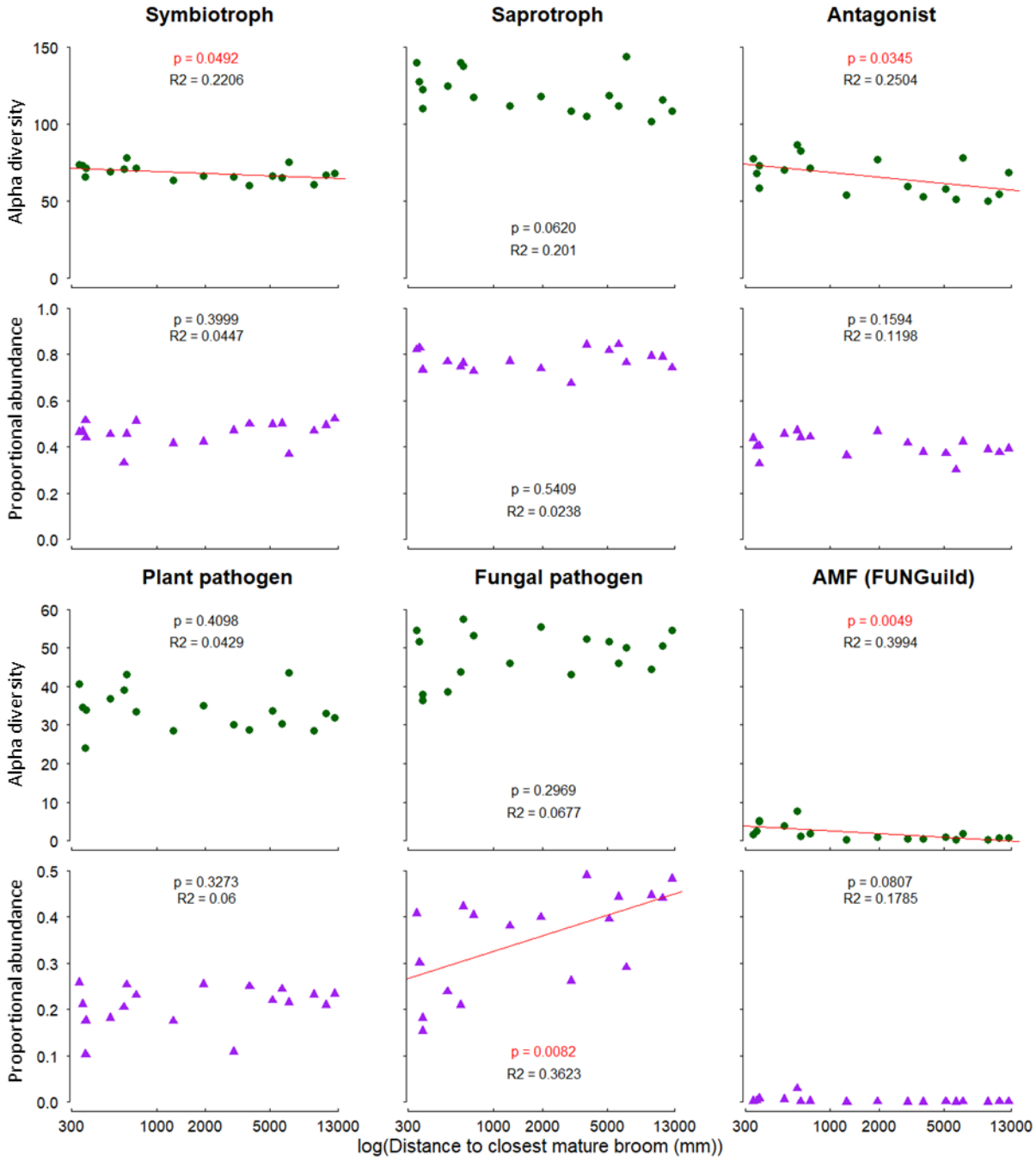
B7. *[Next page; corresponds to Figure 5]* Average alpha diversity (at the level of plots) and proportional abundance of fungal OTUs according to fungal taxa and functional traits over distance from the extracted soil core to the base of the closest mature *C. scoparius* (mm) (note log-transformed axis). Regression lines are shown when $P < 0.05$. OTUs were 'strictly' classified into functional guilds (results with 'loose' classifications, i.e., with overlap between functional guilds, are presented in Appendix C8). *P* and R^2 values are given in the plots.


Ascomycota
All fungi EXCL. Ascomycota
Basidiomycota
Alpha diversity
Proportional abundance
p = 0.3945
R2 = 0.0457
p = 0.0016
R2 = 0.4745
p = 0.0088
R2 = 0.3572
p = 0.1141
R2 = 0.1486
p = 0.1141
R2 = 0.1486
p = 0.1346
R2 = 0.1344
Symbiotroph
Saprotroph
Antagonist
p = 0.2598
R2 = 0.0786
p = 0.0489
R2 = 0.2211
p = 0.0095
R2 = 0.3515
p = 0.1976
R2 = 0.1015
p = 0.5988
R2 = 0.0177
p = 0.4377
R2 = 0.0381
Plant pathogen
Fungal pathogen
Glomeromycotina
p = 0.0239
R2 = 0.28
p = 0.9720
R2 = 1e-04
p = 0.0049
R2 = 0.3994
p = 0.0103
R2 = 0.3453
p = 0.4239
R2 = 0.0404
p = 0.0807
R2 = 0.1785
300
1000
2000
5000
13000
log(Distance to closest mature broom (mm))

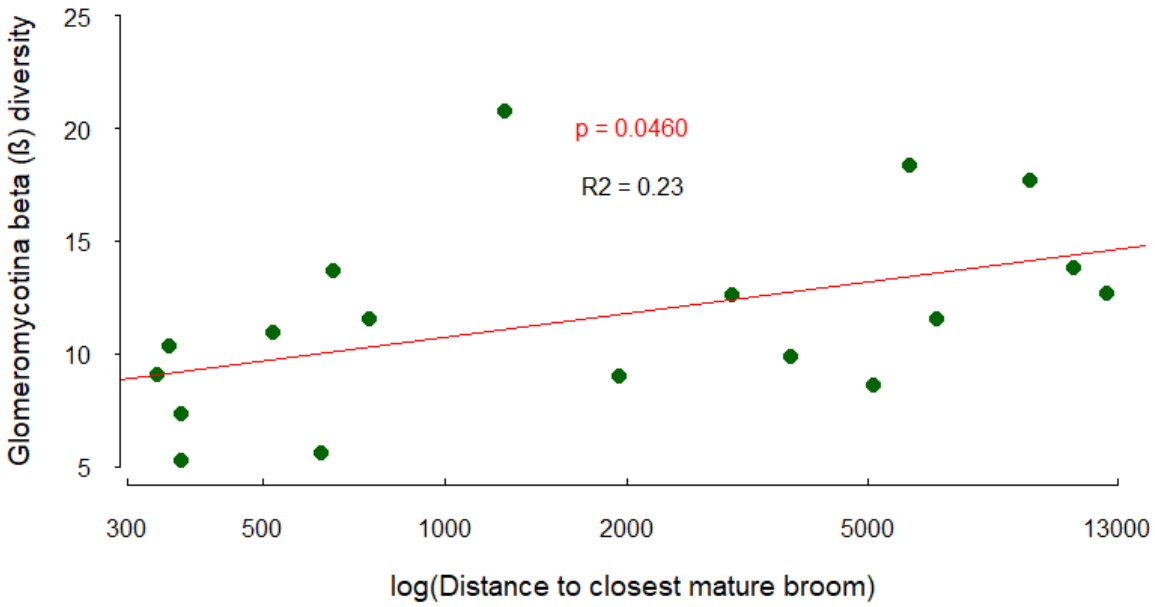
B8. *[Corresponds to Figure 5]* Average alpha diversity (at the level of plots) and proportional abundance of fungal OTUs grouped 'loosely' (i.e., with overlap) according to functional traits over *C. scoparius* coverage (below) and over log-transformed distance from the extracted soil core to the base of the closest mature *C. scoparius* (next page). Regression lines are shown when $P < 0.05$. OTUs for arbuscular mycorrhizal fungi (AMF) according to FUNGuild (Nguyen *et al.* 2016), matched exactly with the OTUs for Glomeromycotina according to the UNITE public database (Nilsson *et al.* 2018). P and R^2 values are given in the plots.

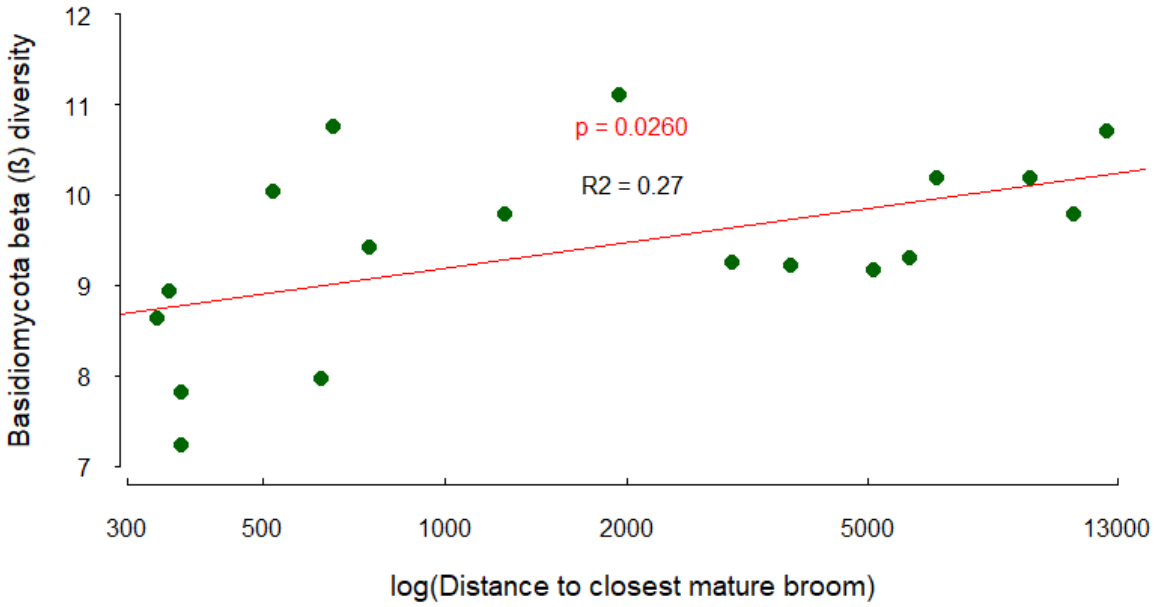


B8. *[Continued]*

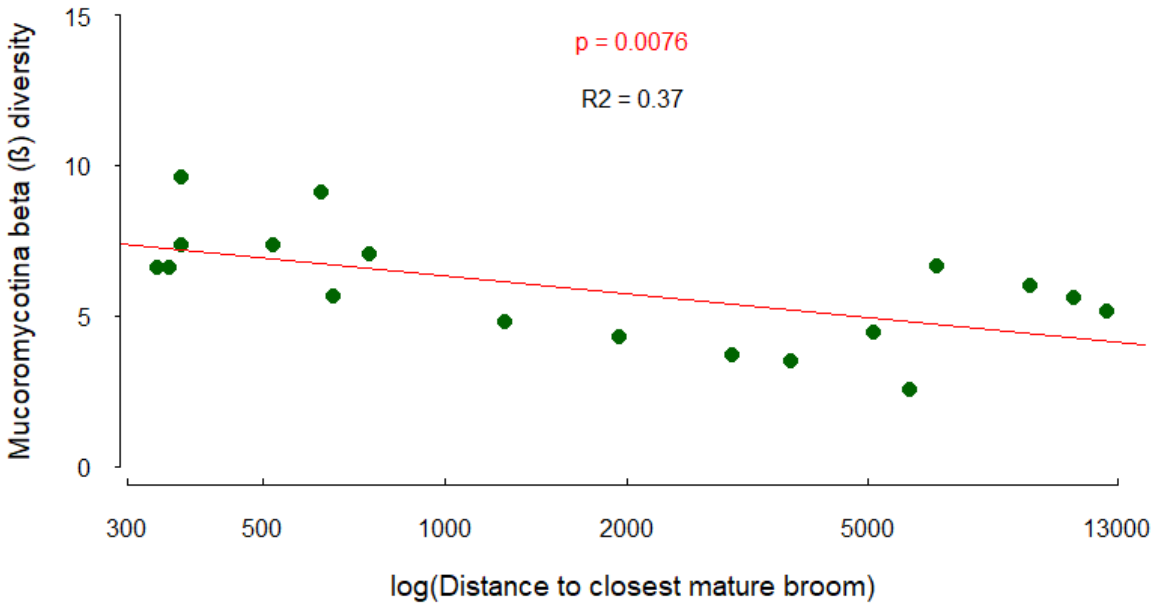


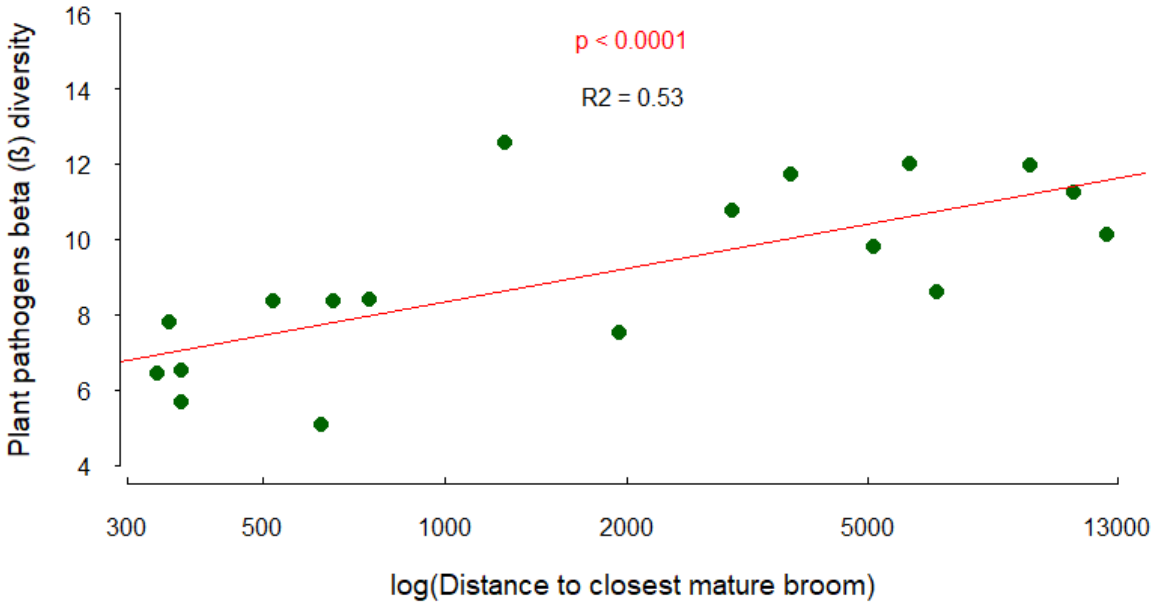
B9. *[Corresponds to Figure 6]* Beta diversity of Glomeromycotina and Basidiomycota (below) and Mucoromycotina and plant pathogens (next page) over log-transformed distance from extracted soil core to closest mature *C. scoparius* (mm). *P* and R^2 values are given in the plots.



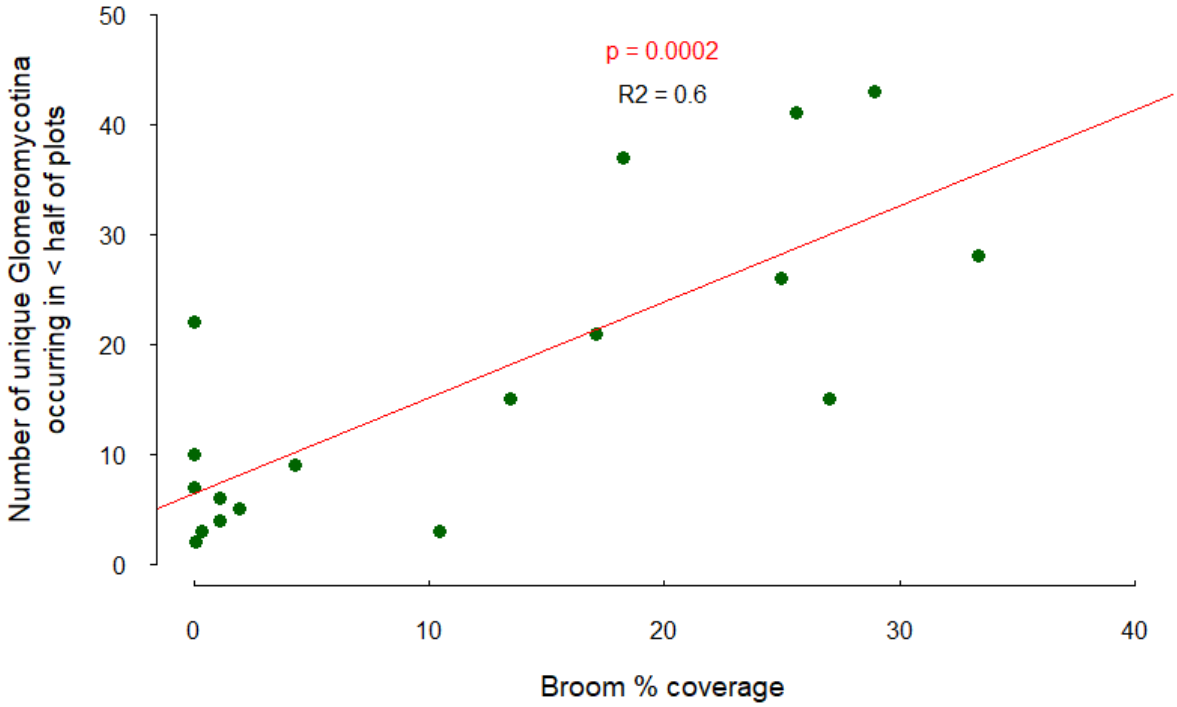


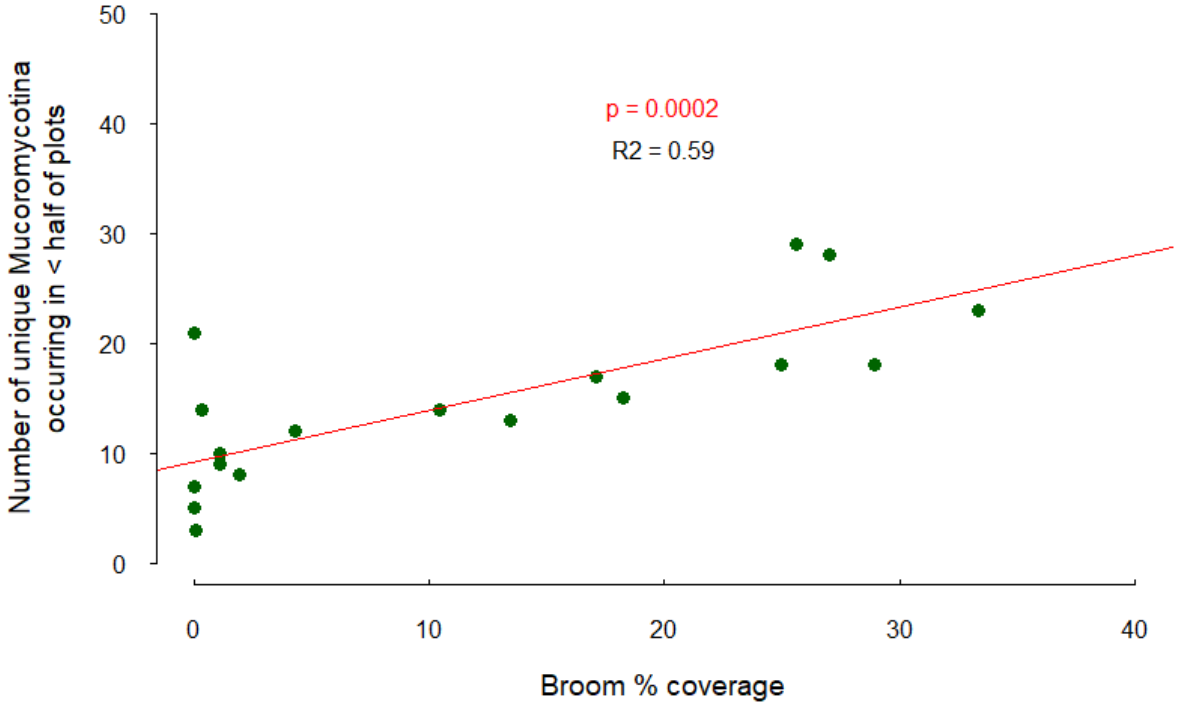
B9. *[Continued]*



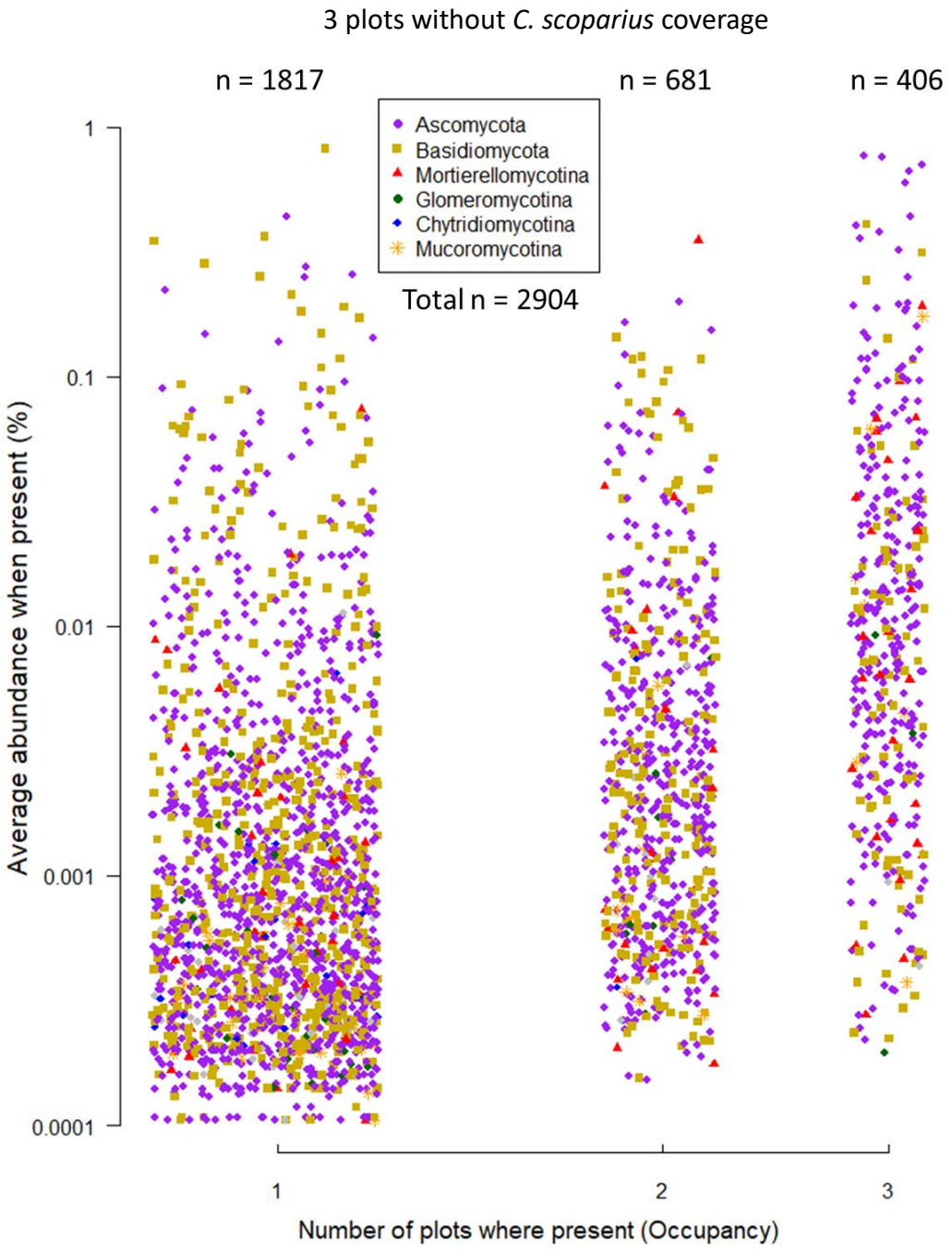


B10. *[Corresponds to Figure 7]* Number of unique Glomeromycotina and Mucoromycotina OTUs occuring in less than half of all plots over *C. scoparius* % coverage per plot. *P* and R^2 values are given in the plots.

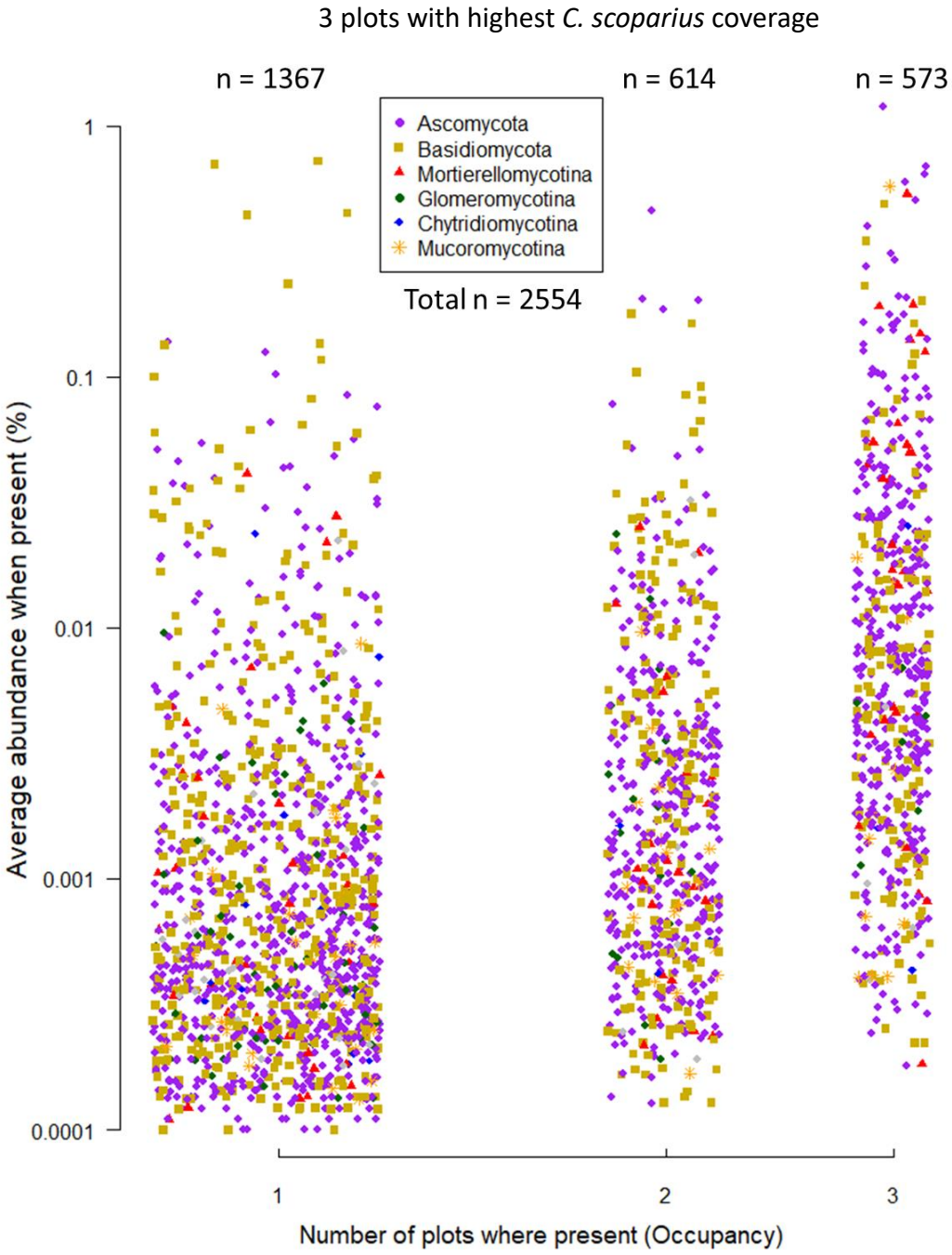




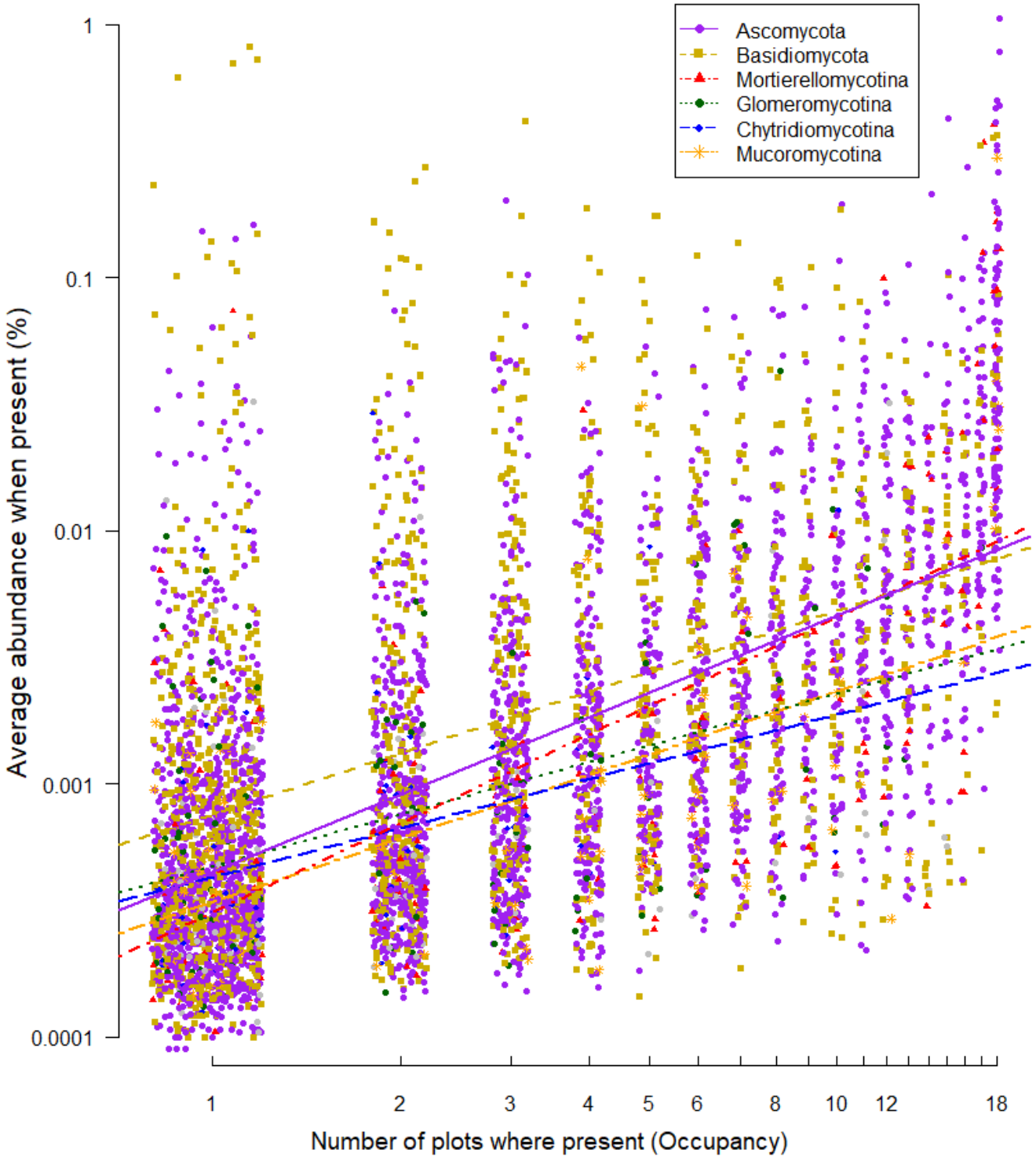
B11. OTU occupancy when present (coloured according to fungal phyla) over number of plots in which the OTU has been detected. Note log-transformed x and y axes. The number of OTUs present accoruding to the number of plots is given above each column. There were 41.3% more OTUs (n = 167) found across all three plots with highest *C. scoparius* coverage compared to across all three plots with lowest *C. scoparius* coverage. The three plots with highest *C. scoparius* coverage (below) were MW20, MW24 & MW29. The three plots without *C. scoparius* coverage (next page) were MW13, MW19 & MW26.



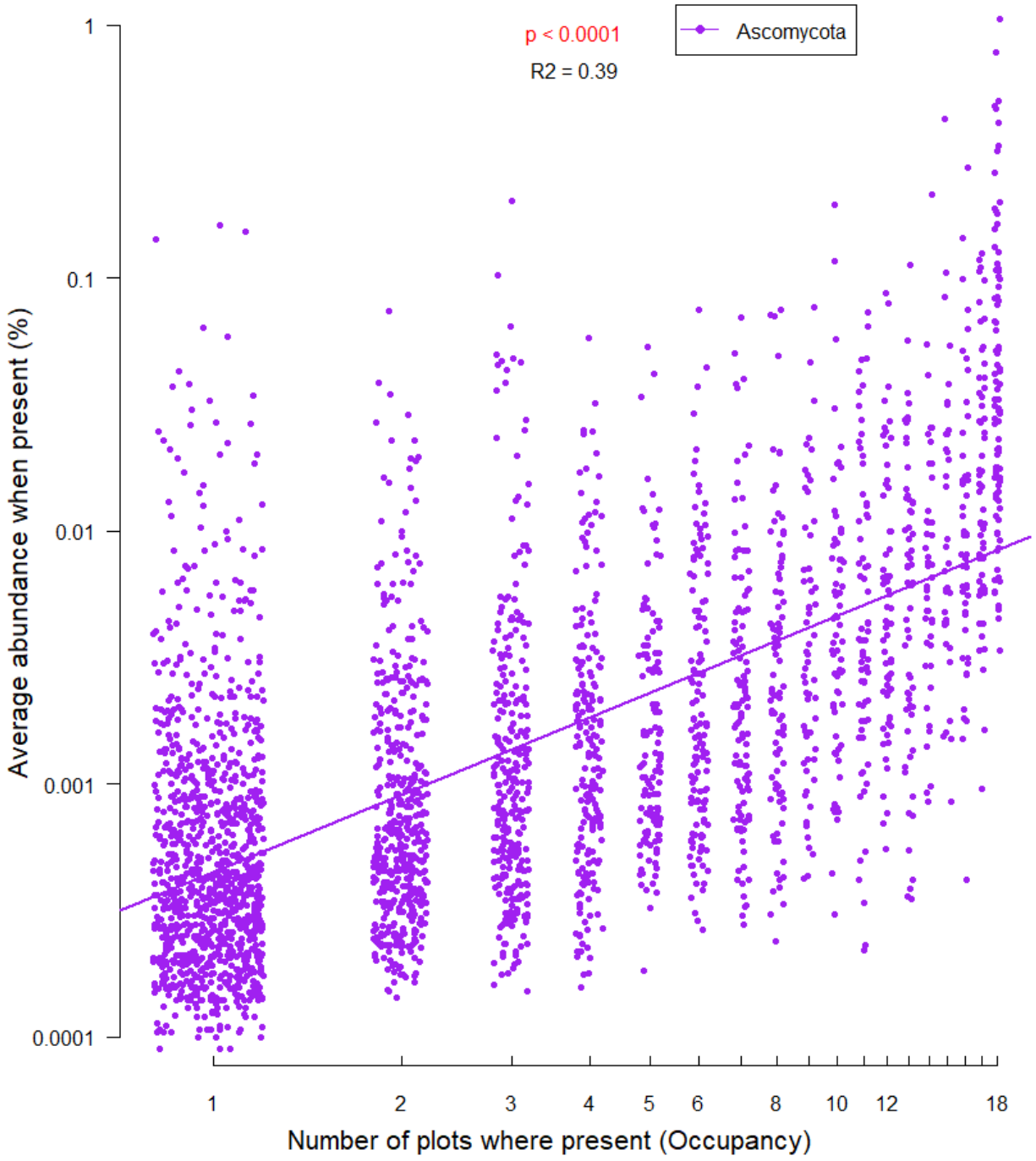
B11. *[Continued]*



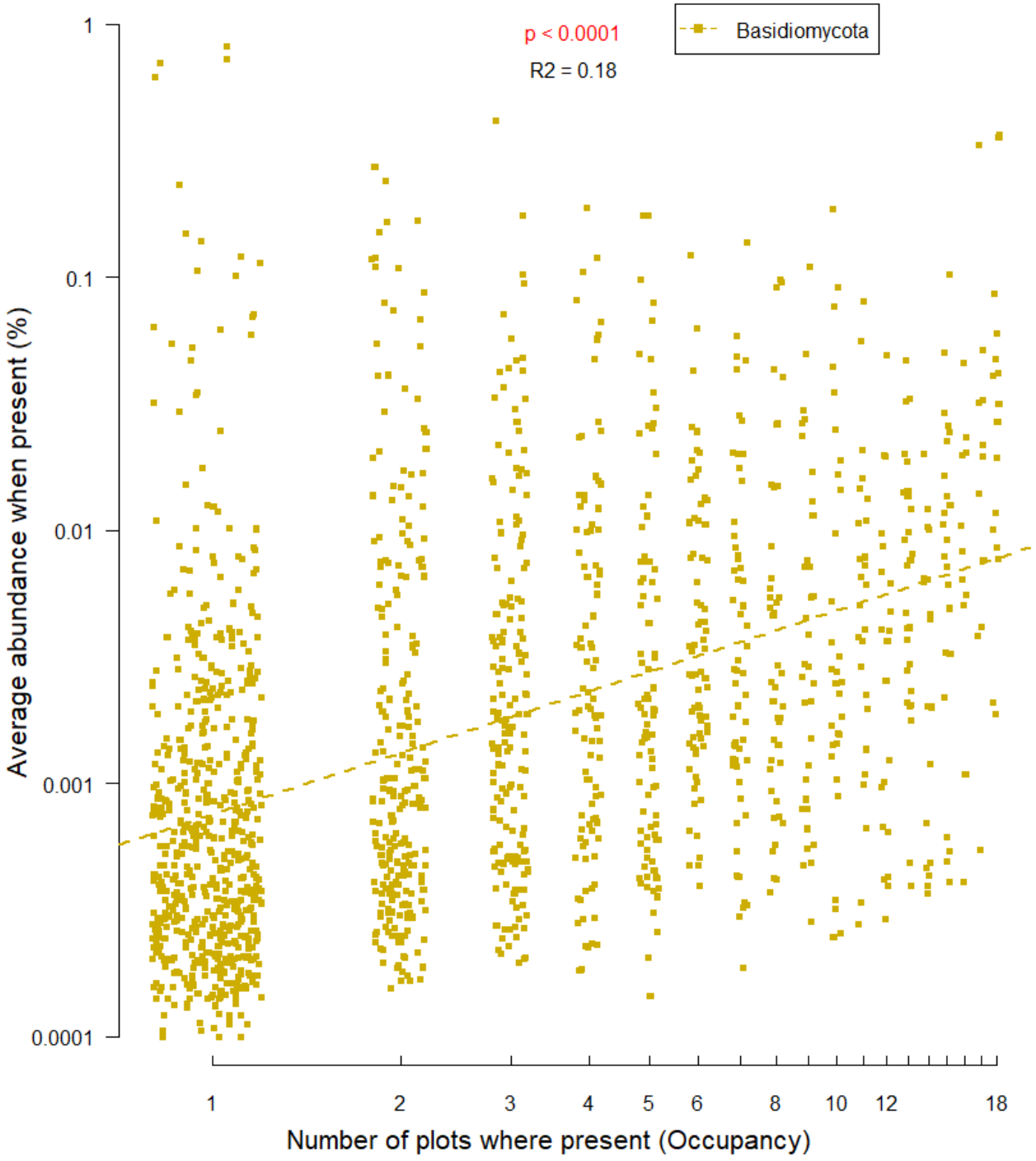
B12. OTU occupancy when present for all fungal taxa and for individual fungal taxa over number of plots in which the OTU has been detected. Note log-transformed x and y axes.



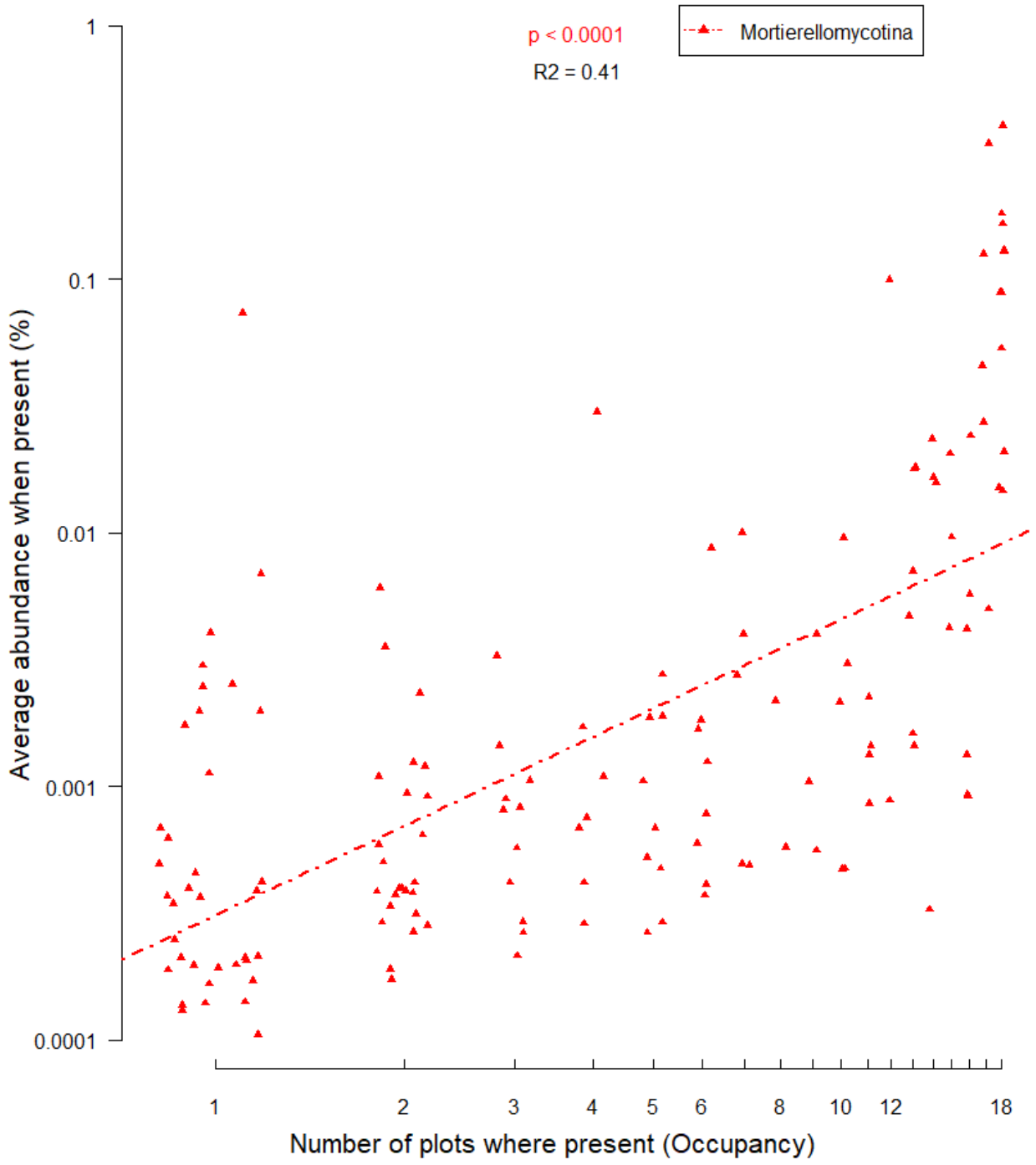
B12. *[Continued]*



B12. *[Continued]*

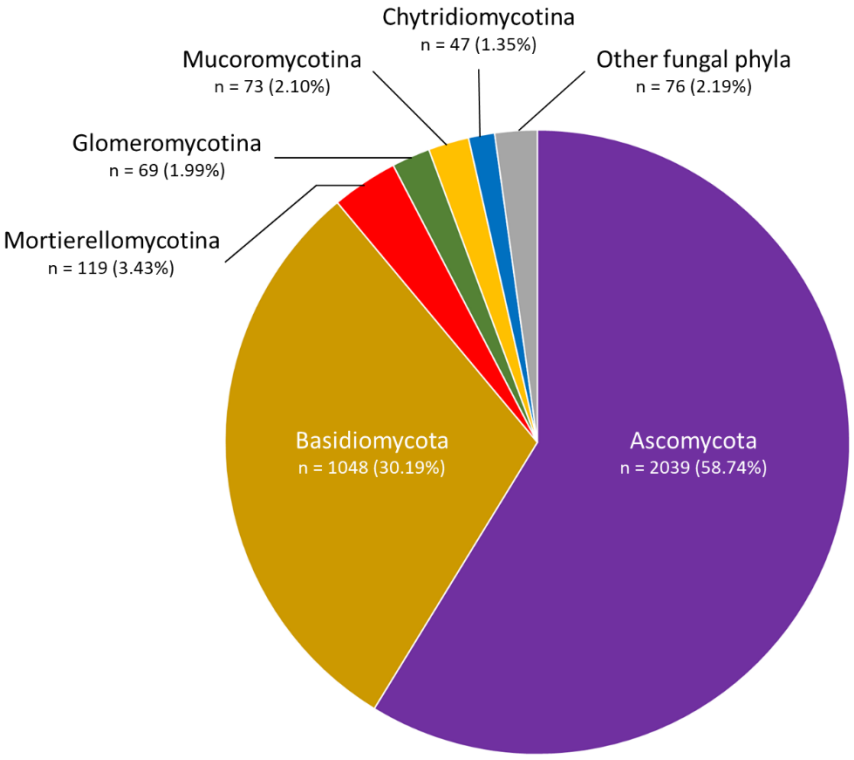


B12. *[Continued]*



Appendix C (Supplement Chapter 4)

C1. All fungal OTUs for both stand-alone and pooled samples split according to taxonomic phyla. Current taxonomy places Glomeromycotina into Mucoromycota, yet due to database limitations, Glomeromycotina follows its older taxonomy of Glomeromycota.



C2. Mean number of fungal OTUs (not rarefied) for each fungal phyla according to the number of samples in a pool. eDNA samples which had less OTUs than stand-alone samples are highlighted in blue and eDNA samples with more OTUs than standaone samples are in green.

		Both pooled and stand-alone eDNA samples	Stand-alone eDNA samples	Pooled eDNA samples	eDNA samples pooled by			
					3	6	12	24
	Number of samples	233	143(*)	90	48	24	12	6
Mean number of fungal OTUs	All fungal phyla	208.219	206.615	210.767	**186.250**	220.792	257.750	272.833
	Ascomycota	**134.622**	140.406	**125.433**	**110.750**	**133.750**	151.500	157.500
	Basidiomycota	46.279	40.517	55.433	47.125	56.708	72.917	81.833
	Glomeromycota	**1.927**	2.147	**1.578**	**1.542**	**1.417**	**1.917**	**1.833**
	Mortierellomycotina	17.717	15.573	21.122	20.083	21.417	23.500	23.500
	Chytridiomycotina	**0.760**	0.797	**0.700**	**0.750**	**0.625**	**0.583**	0.833
	Mucoromycotina	**5.236**	5.287	**5.156**	**4.958**	5.417	**5.250**	5.500

(*) 144 stand-alone eDNA samples underwent sequencing, yet one was ommitted due to having < 1000 reads

C3. *[Corresponds to Figures 3 & 4]* Linear mixed-effect model estimates for rarefied richness by fungal phylum over log-transformed number of samples per pool (t-values outside of parentheses, p-values within parentheses).

	Pooled	log(samples in pool)	Pooled × log(samples in pool)
Rarefied richness of all fungal phyla	-3.16 (< 0.0001)	28.541 (< 0.0001)	-4.568 (< 0.0001)
Rarefied richness of Ascomycota	-4.291 (< 0.0001)	25.862 (< 0.0001)	-5.368 (< 0.0001)
Rarefied richness of Basidiomycota	-2.929 (< 0.0001)	40.855 (< 0.0001)	-9.345 (< 0.0001)
Rarefied richness of Mortierellomycotina	1.94 (< 0.0001)	30.007 (< 0.0001)	-9.531 (< 0.0001)

C4. *[Corresponds to Figure 5]* Linear mixed-effect model estimates for proportional abundance by fungal phylum over log-transformed number of samples per pool (t-values outside of parentheses, p-values within parentheses).

	Pooled	log(samples in pool)	Pooled × log(samples in pool)
Proportional abundance of Ascomycota	-10.605 (< 0.0001)	-0.017 (0.7399)	0.444 (0.6569)
Proportional abundance of Basidiomycota	4.646 (< 0.0001)	0.011 (0.8601)	-0.24 (0.8107)
Proportional abundance of Mortierellomycotina	9.619 (< 0.0001)	0.009 (0.8629)	-0.232 (0.8167)

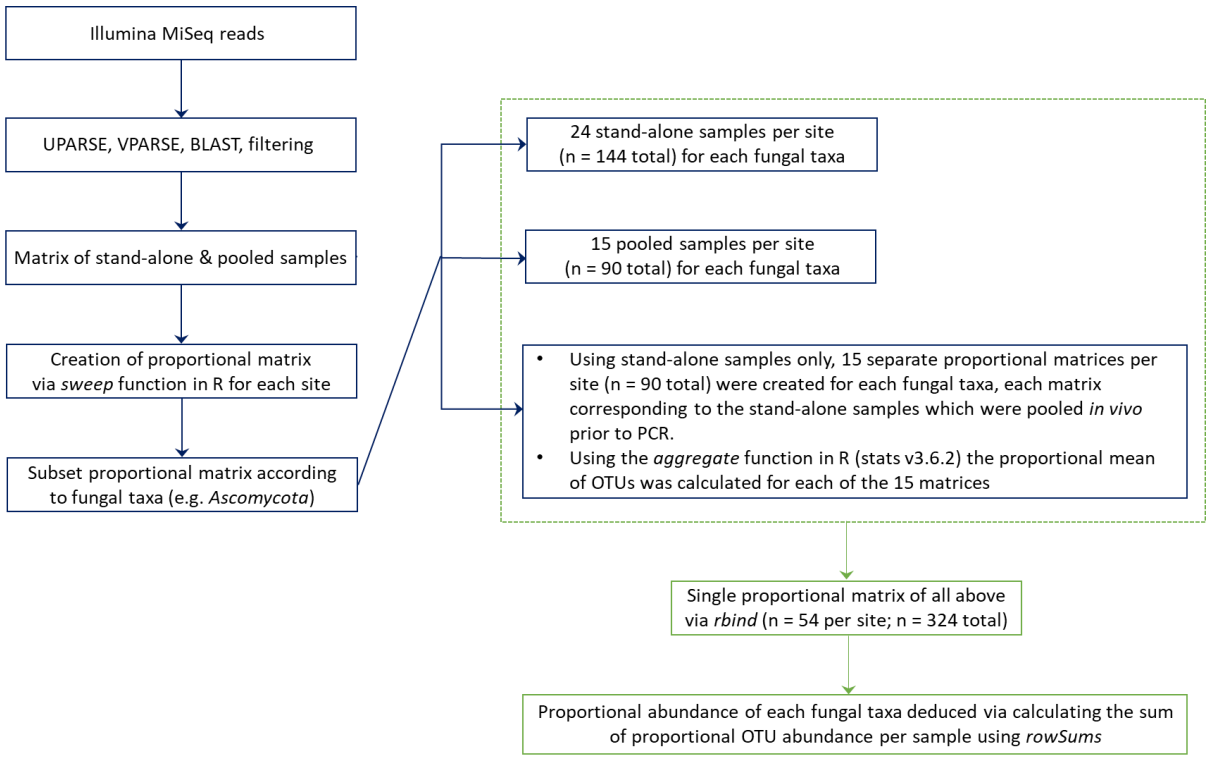
C5. *[Corresponds to Figure 7]* Linear mixed-effect model estimates for rarefied richness over mean *C. scoparius* % coverage for all fungal phyla and for individual fungal phylum (t-values outside of parentheses, p-values within parentheses).

	Broom coverage	Samples in pool	Pooled	Broom coverage × samples in pool	Samples in pool × pooled
Rarefied richness of all fungal phyla	1.821 (0.02558)	12.488 (< 0.0001)	0.469 (< 0.0001)	2.188 (0.0287)	-7.564 (< 0.0001)
Rarefied richness of Ascomycota	1.852 (0.064)	17.848 (< 0.0001)	- 2.324 (< 0.0001)	.	-7.781 (< 0.0001)
Rarefied richness of Basidiomycota	4.903 (0.018343)	17.129 (< 0.0001)	0.754 (< 0.0001)	2.583 (0.009808)	-11.902 (< 0.0001)
Rarefied richness of Mortierellomycotina	0.08 (0.44781)	11.939 (< 0.0001)	3.539 (< 0.0001)	2.418 (0.01559)	-10.076 (< 0.0001)

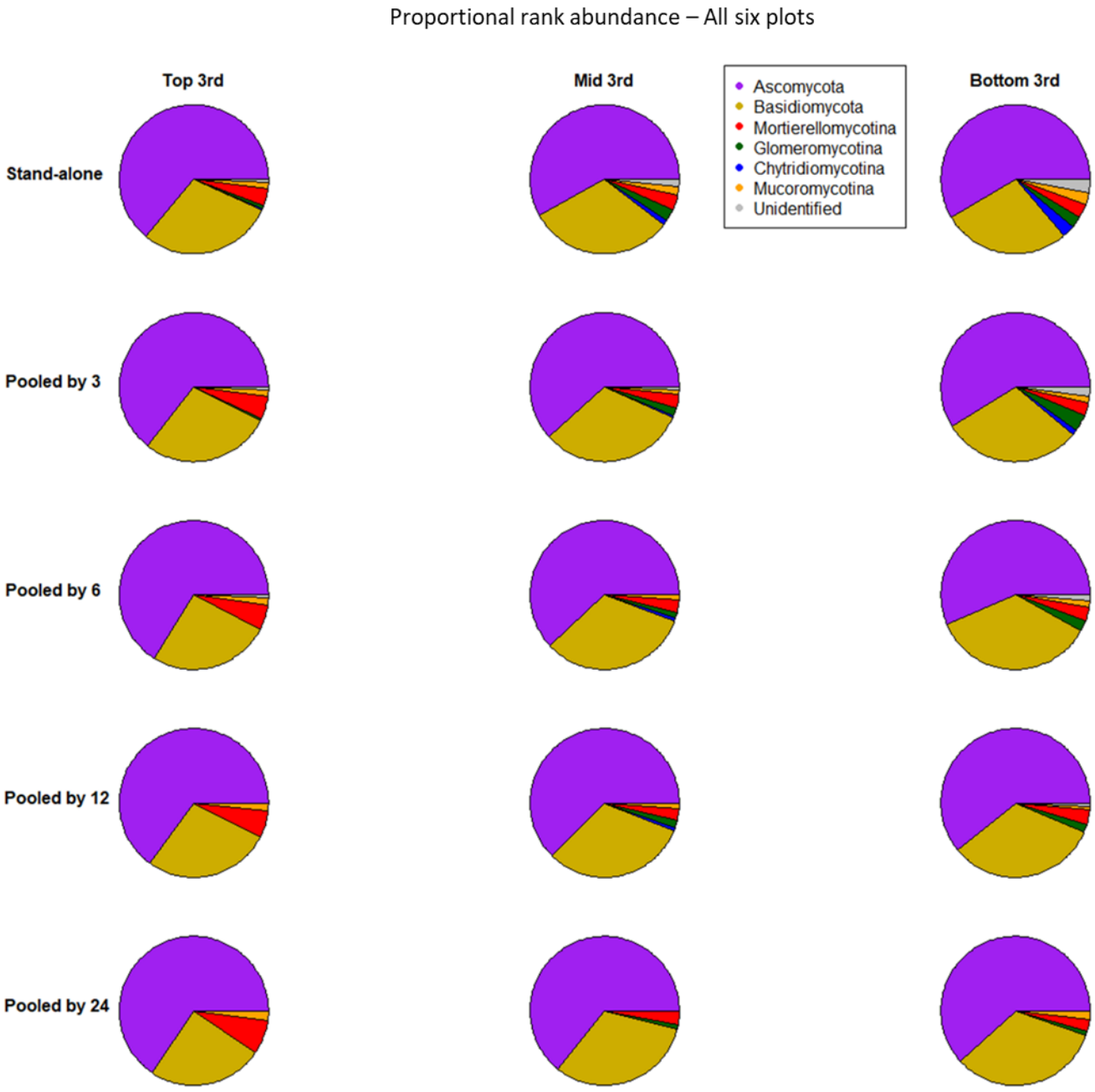
C6. *[Corresponds to Figure 8]* Linear mixed-effect model estimates for rarefied richness of individual fungal phyla in proportion to the rarefied richness of all fungal phyla over mean *C. scoparius* % (t-values outside of parentheses, p-values within parentheses).

Rarefied richness of	Broom coverage	Samples in pool	Pooled	Broom coverage × samples in pool	Broom coverage × pooled	Samples in pool × pooled	Broom coverage × samples in pool × pooled
Basidiomycota ~ of all fungal phyla	1.08 (0.294)	12.262 (< 0.0001)	0.04 (< 0.0001)	-2.288 (0.205)	0.662 (0.00382)	-6.632 (< 0.0001)	2.083 (0.0372)
Mortierellomycotina ~ all fungal phyla	-2.317 (0.04234)	0.899 (0.80651)	4.755 (0.01272)	1.683 (0.09242)	.	-4.281 (< 0.0001)	.
Ascomycota ~ all fungal phyla	0.091 (0.9277)	1.619 (0.1055)	-17.759 (< 0.0001)	.	.	.	.

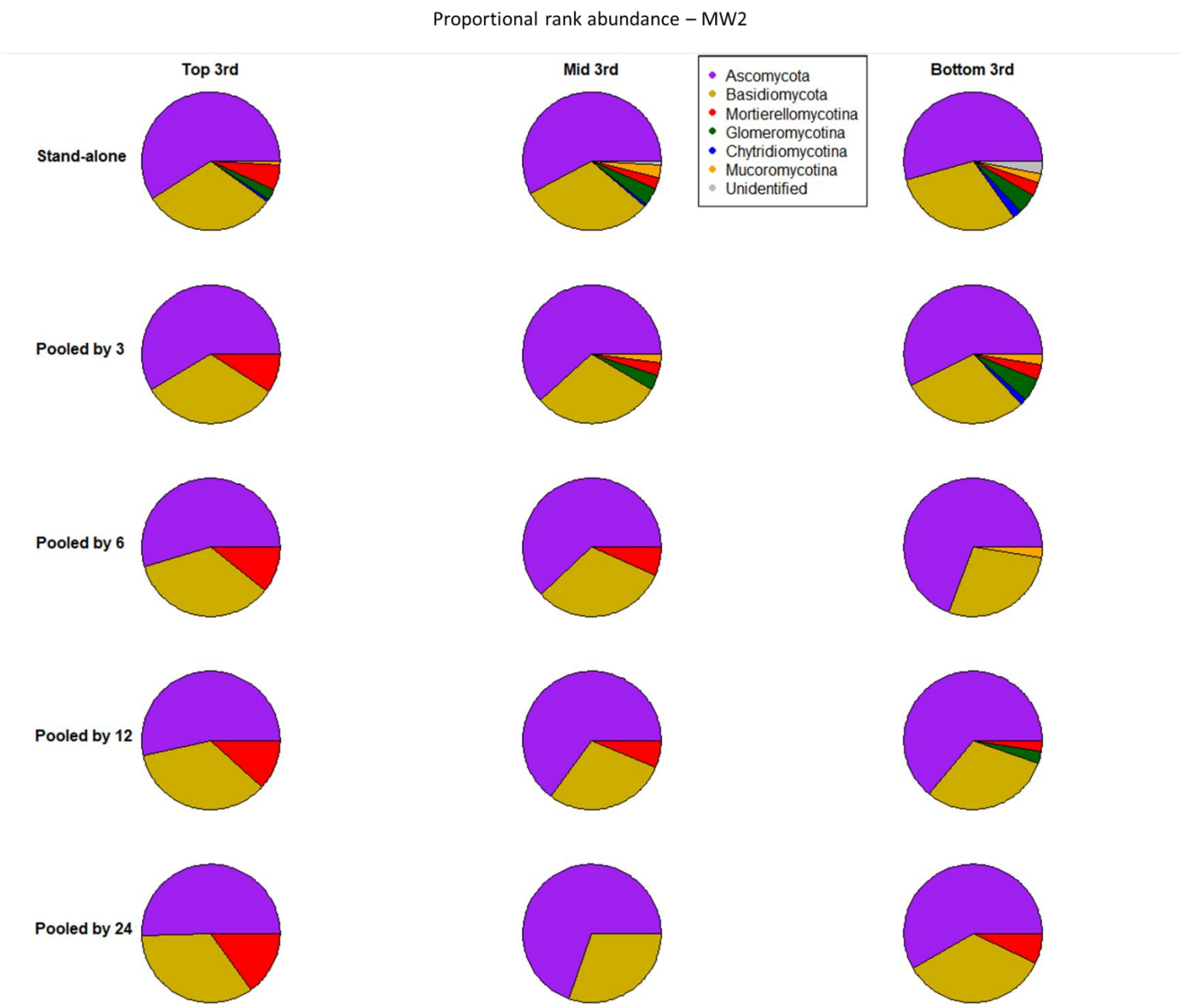
C7. Flowchart for calculated proportional abundance without rarefaction.



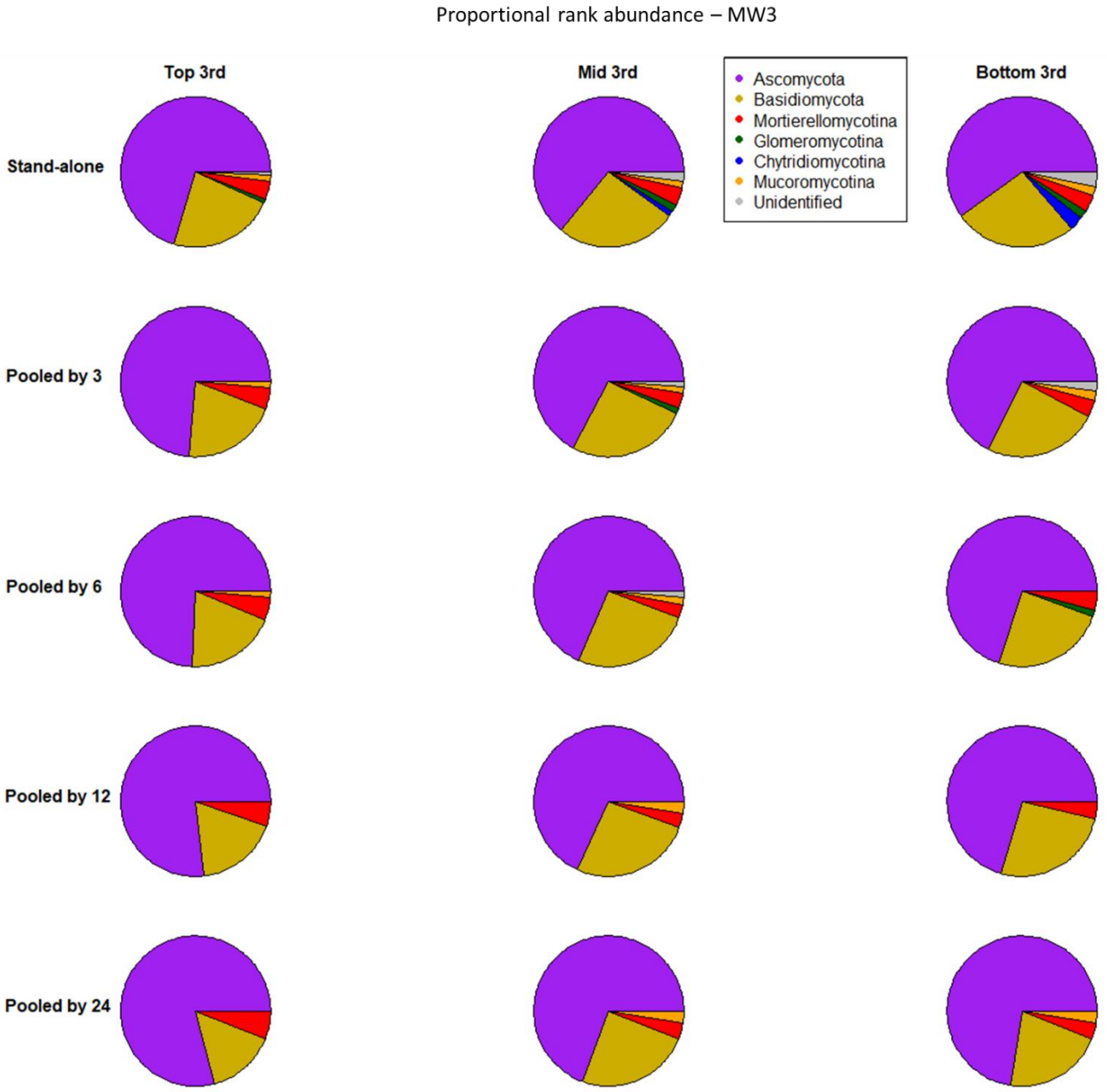
C8. Taxonomic composition of OTUs from across all plots (below) and for each individual plot (next page), split by the top 3rd, middle 3rd and bottom 3rd proportional rank abundance percentile for each degree of pooling.



C8. *[Continued]*



C8. *[Continued]*



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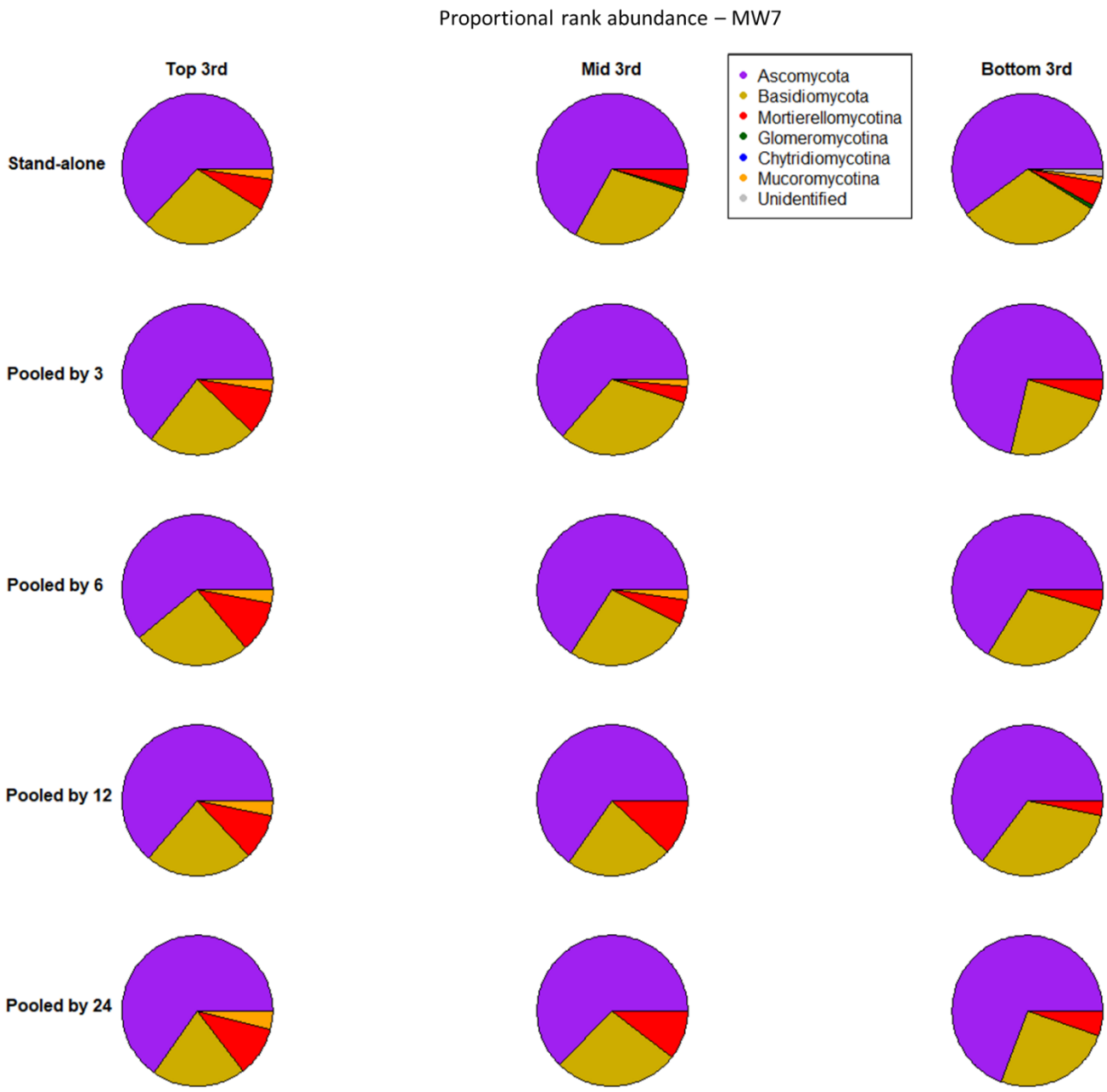

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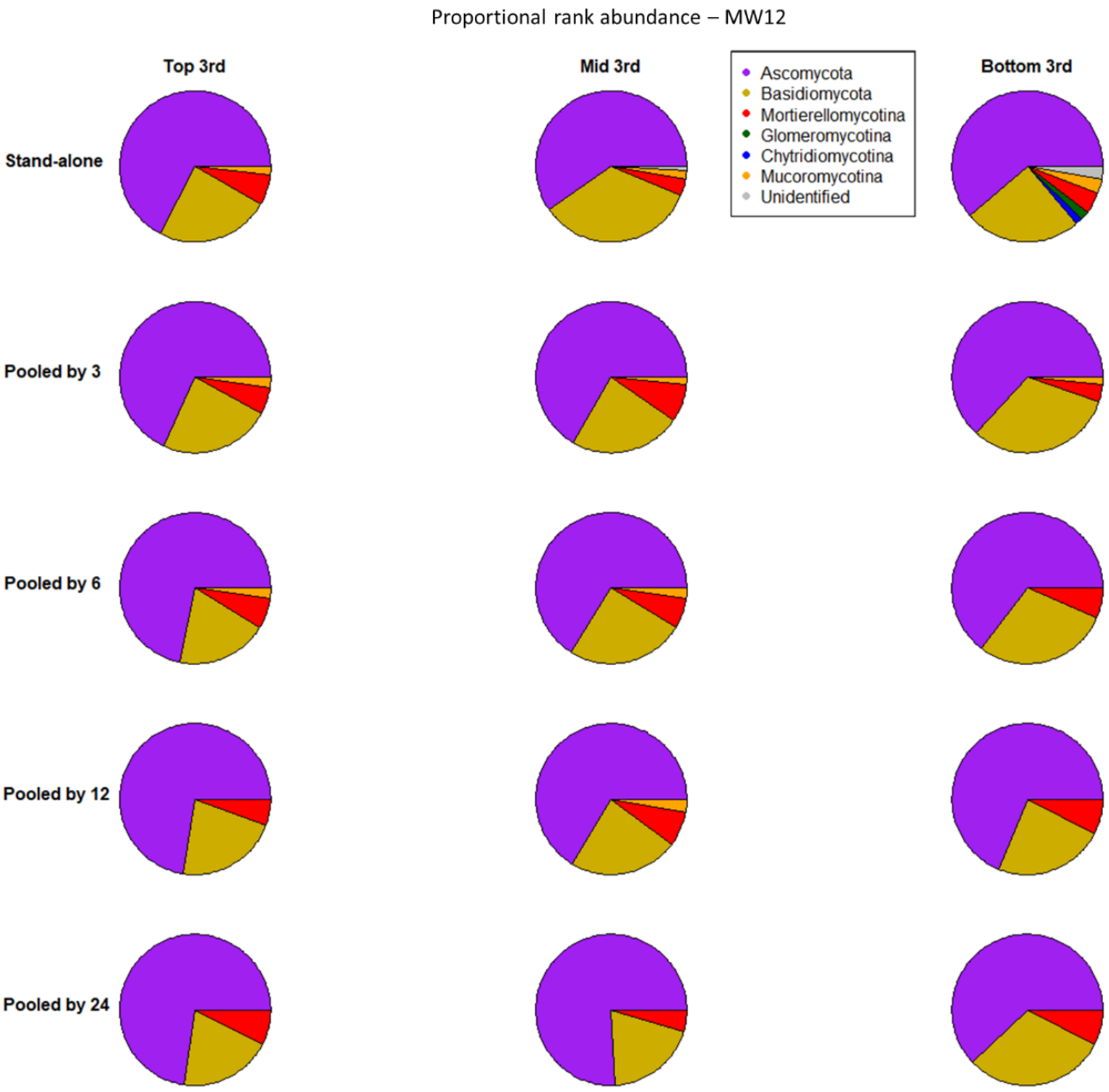

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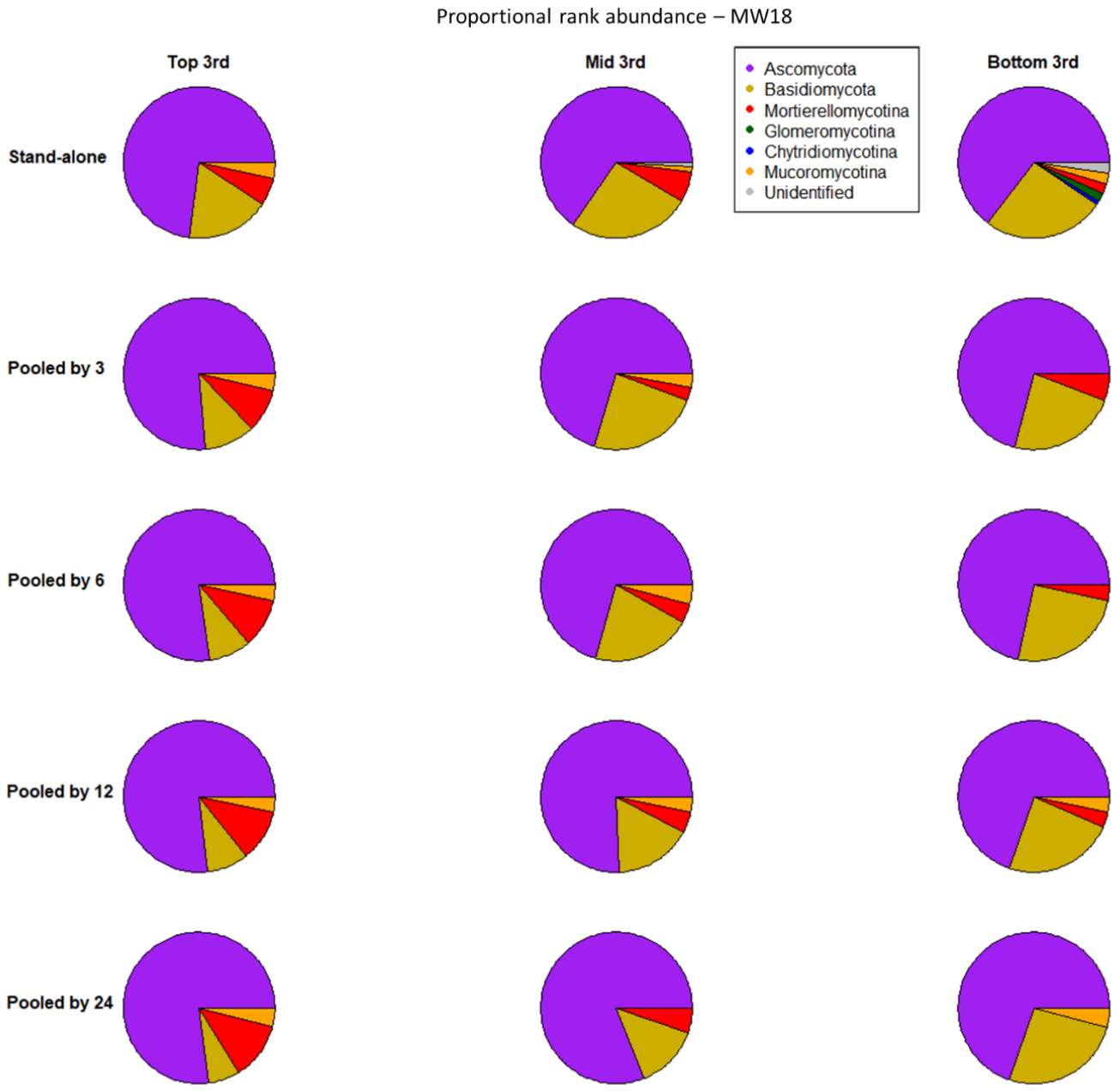
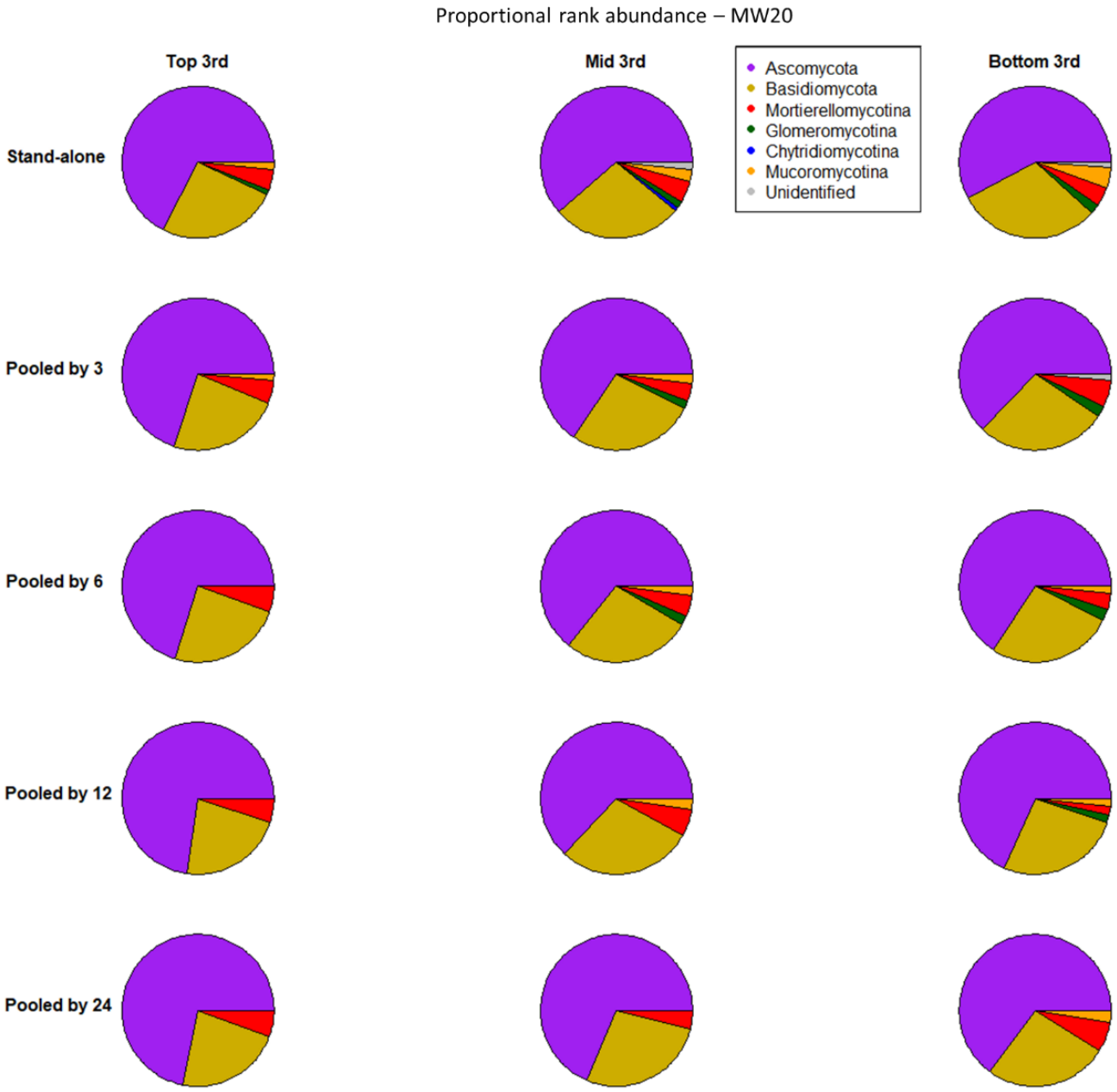
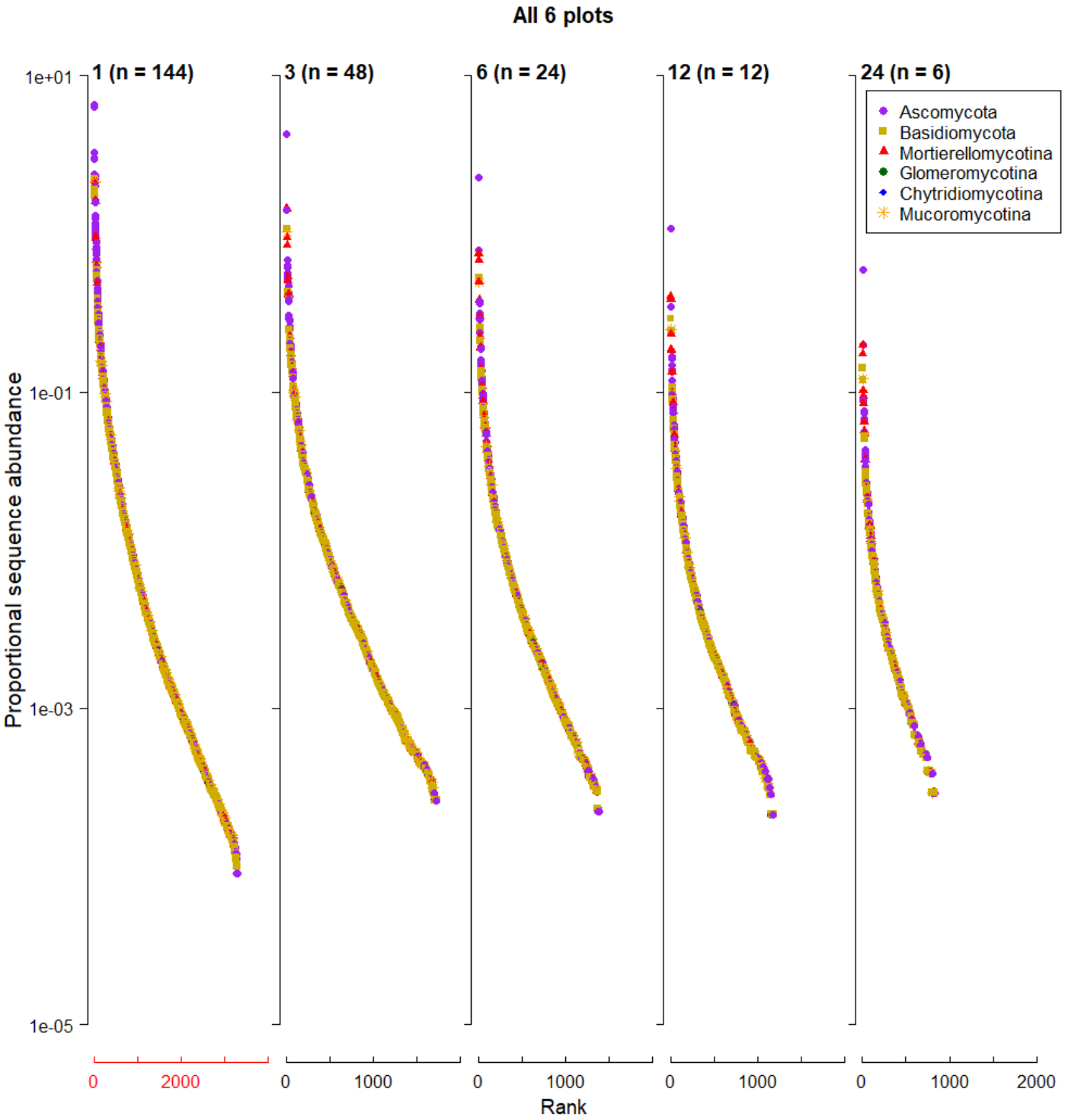


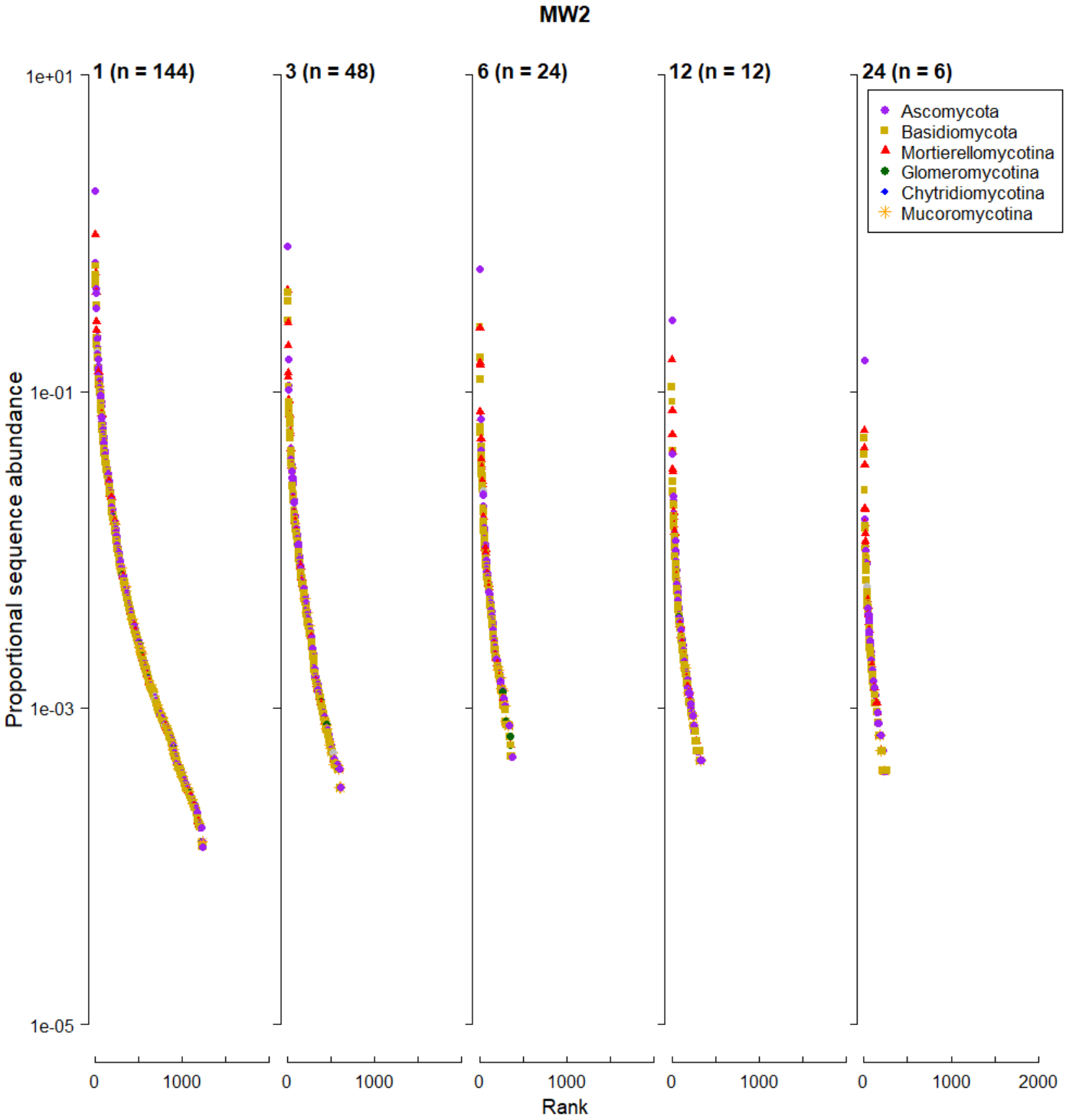
C8. *[Continued]*



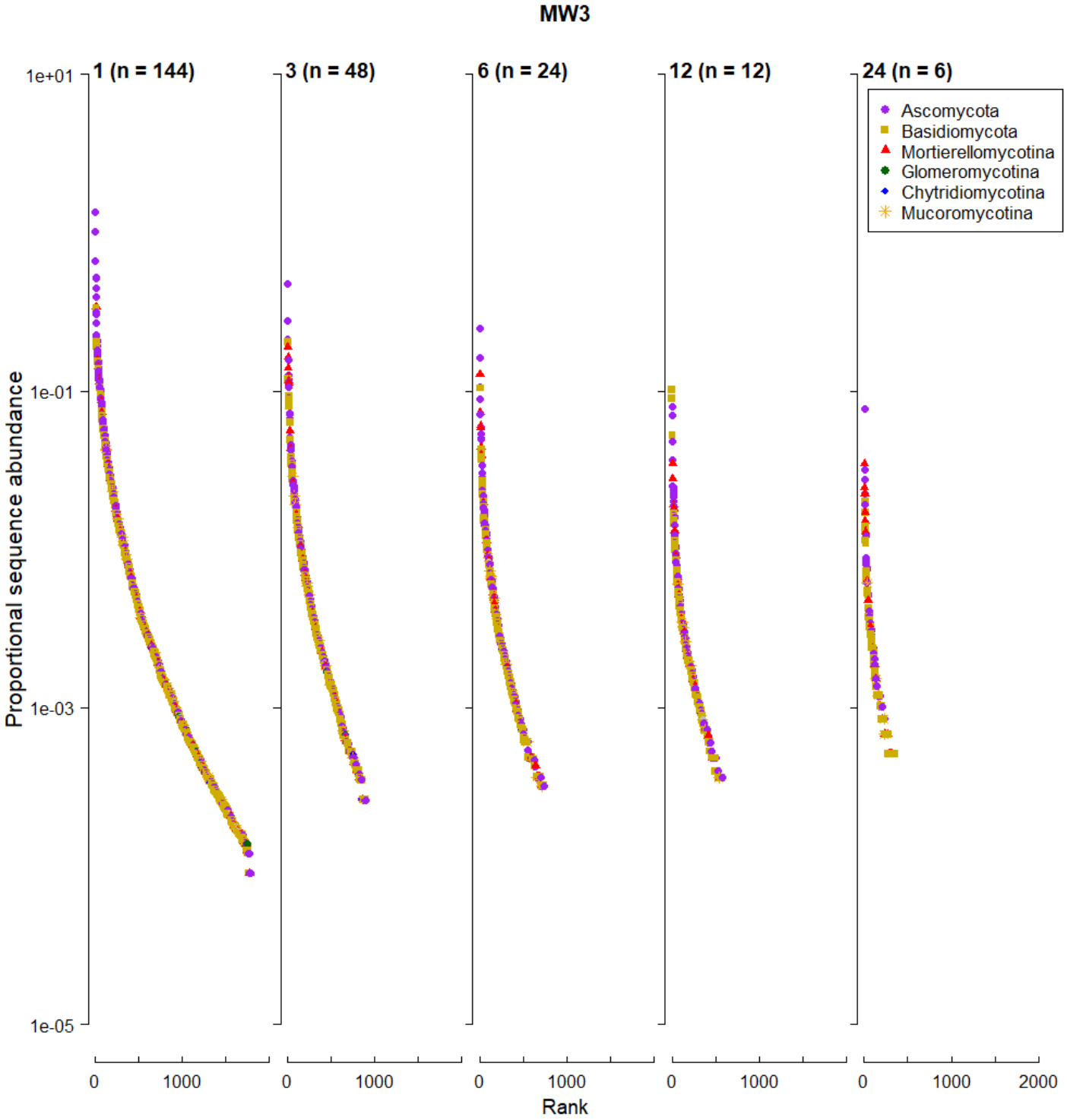
C9. Proportional rank sequence abundance across all plots for each degree of pooling (3, 6, 12 & 24). The y-axis of the stand-alone samples (in red) is set to double that of the rest.



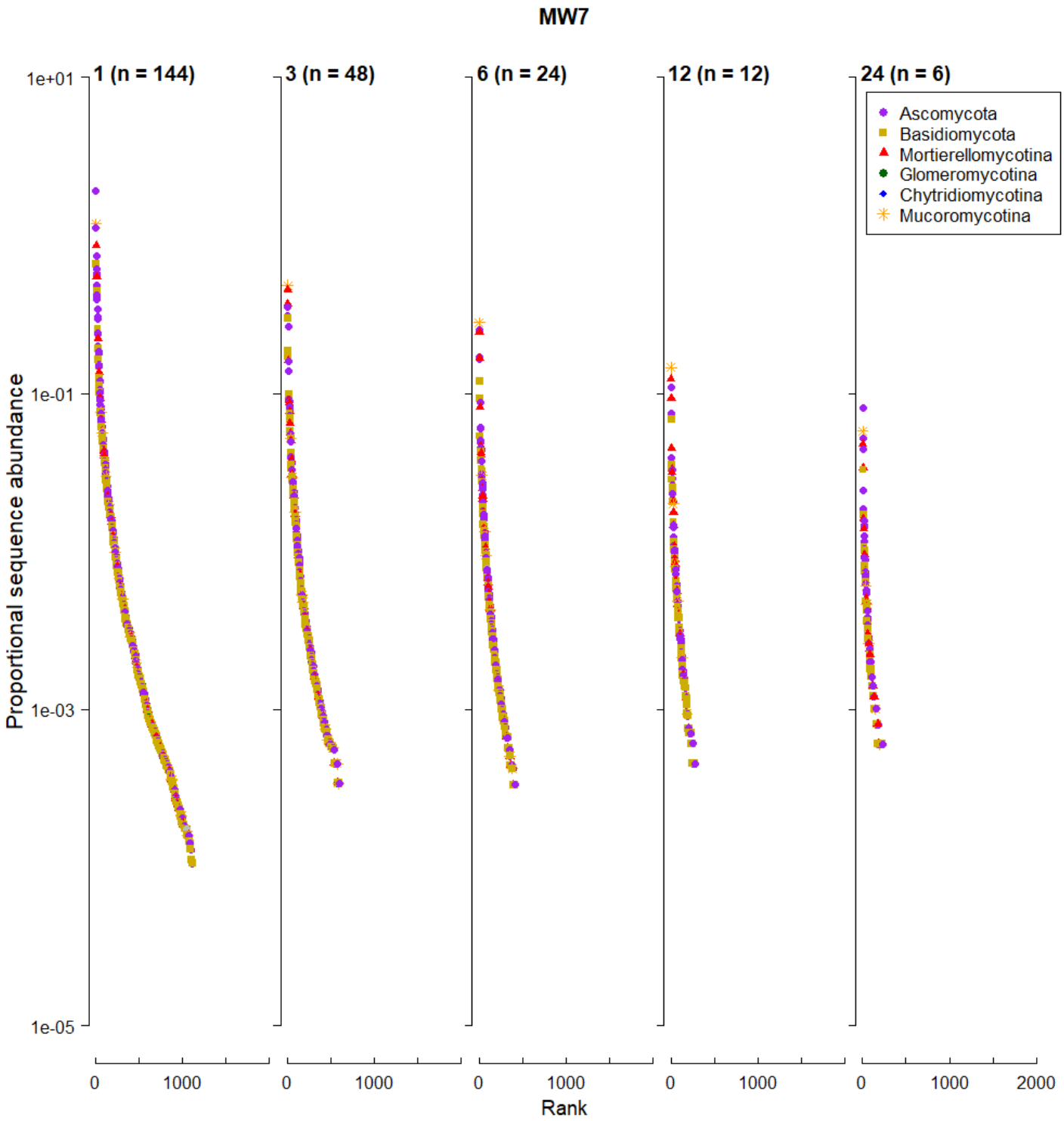
C10. Proportional rank sequence abundance for each plot for each degree of pooling (3, 6, 12 & 24).



C10. *[Continued]*



C10. *[Continued]*



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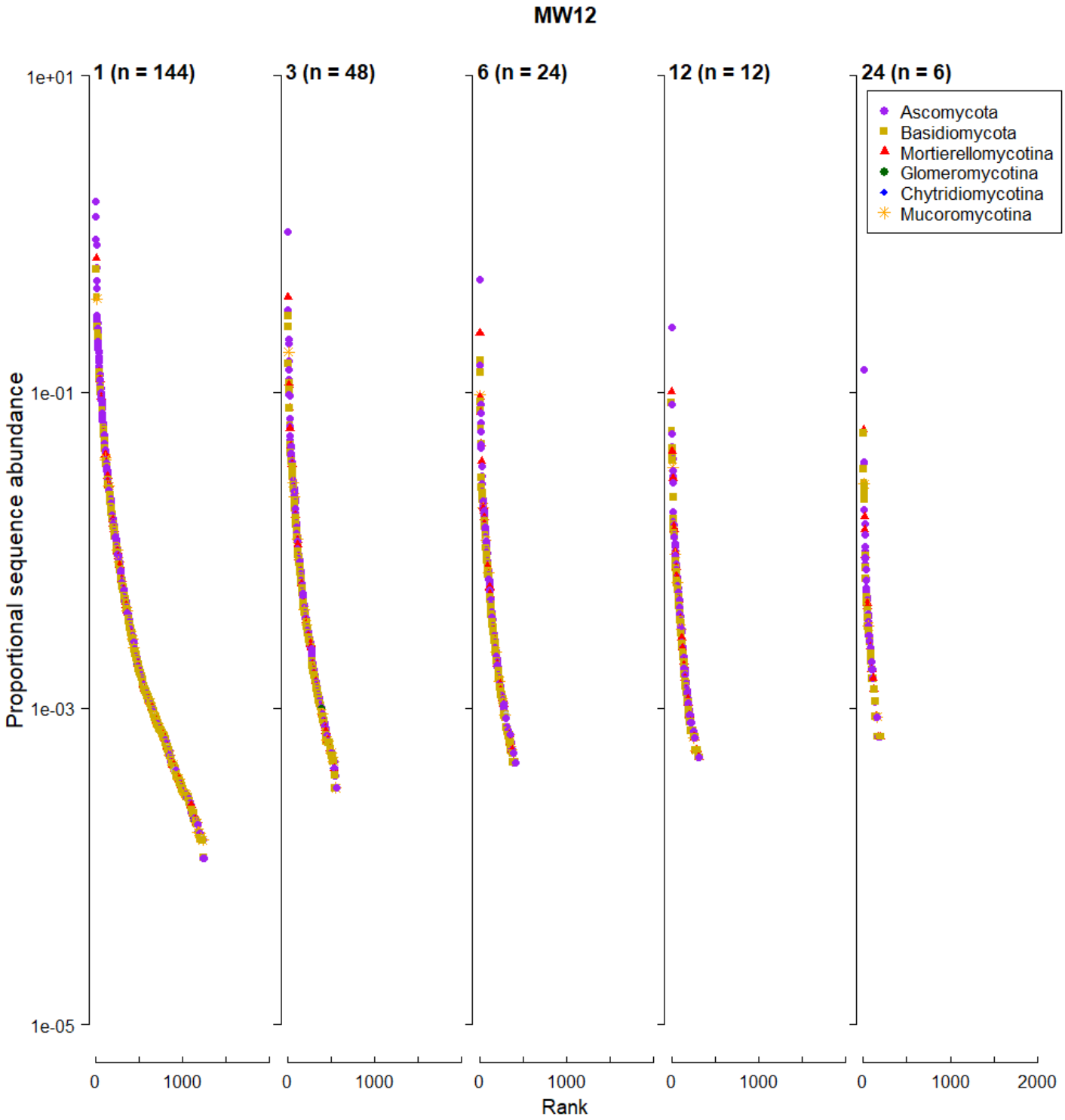
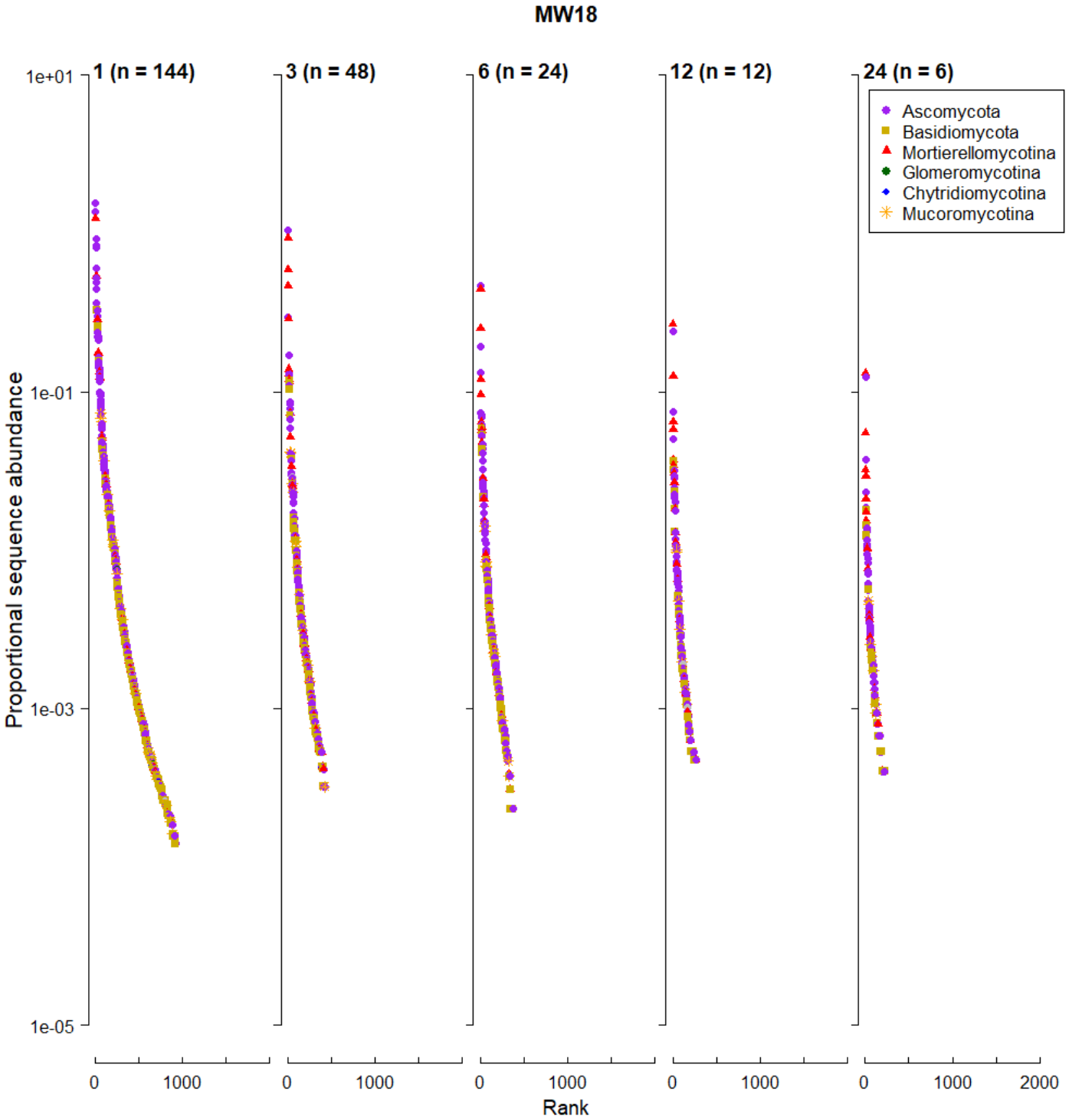
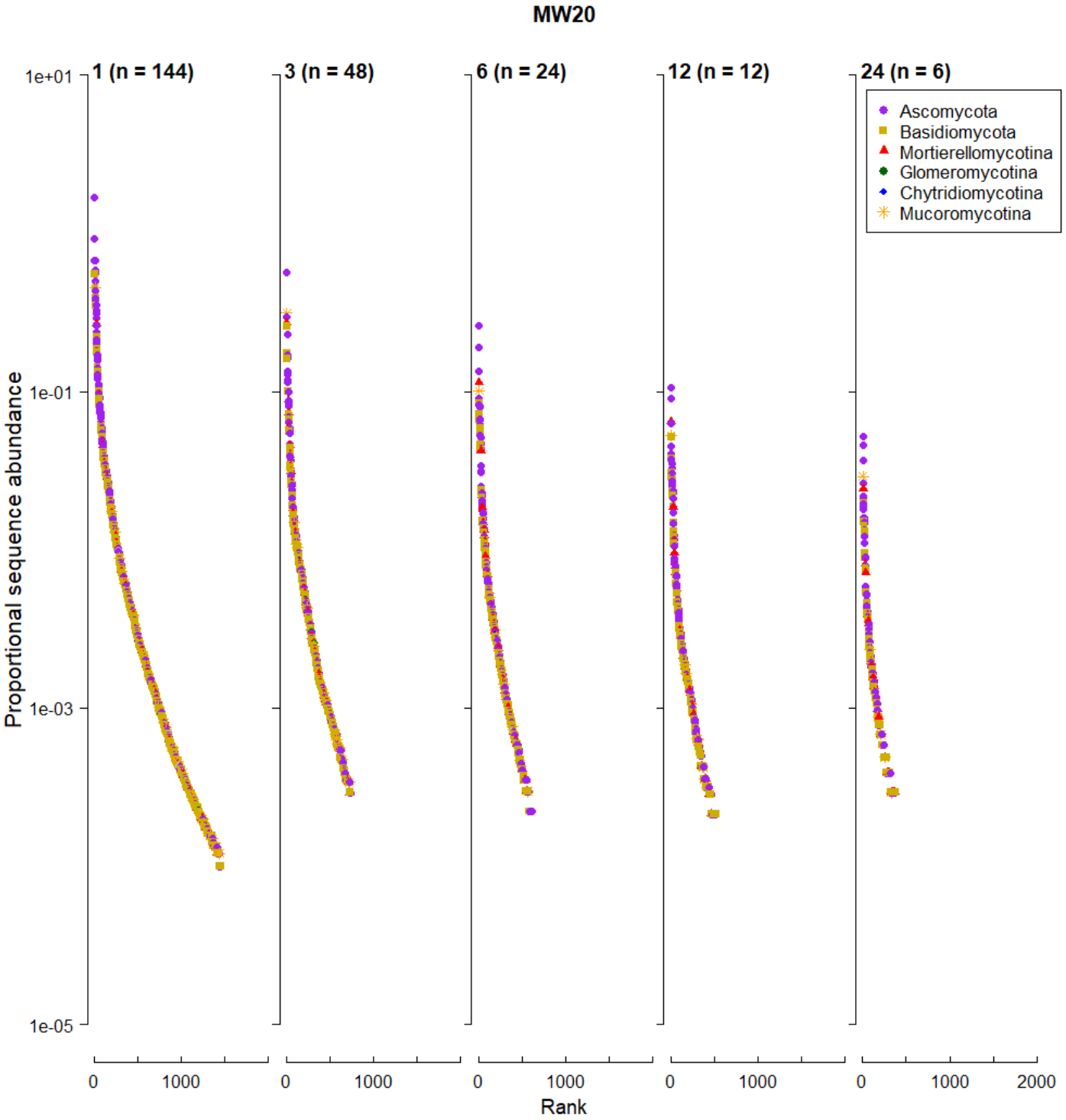


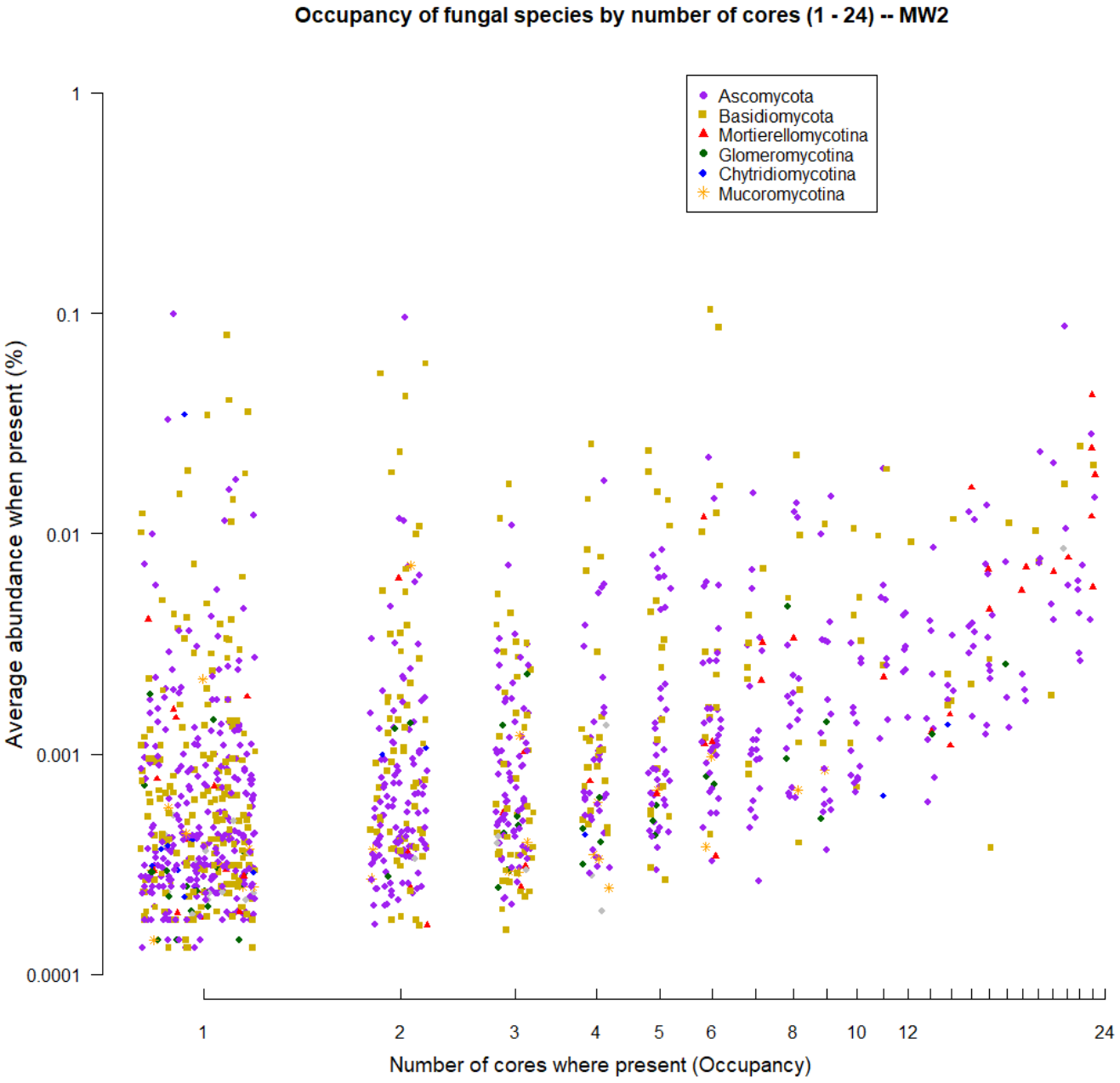
C10. *[Continued]*



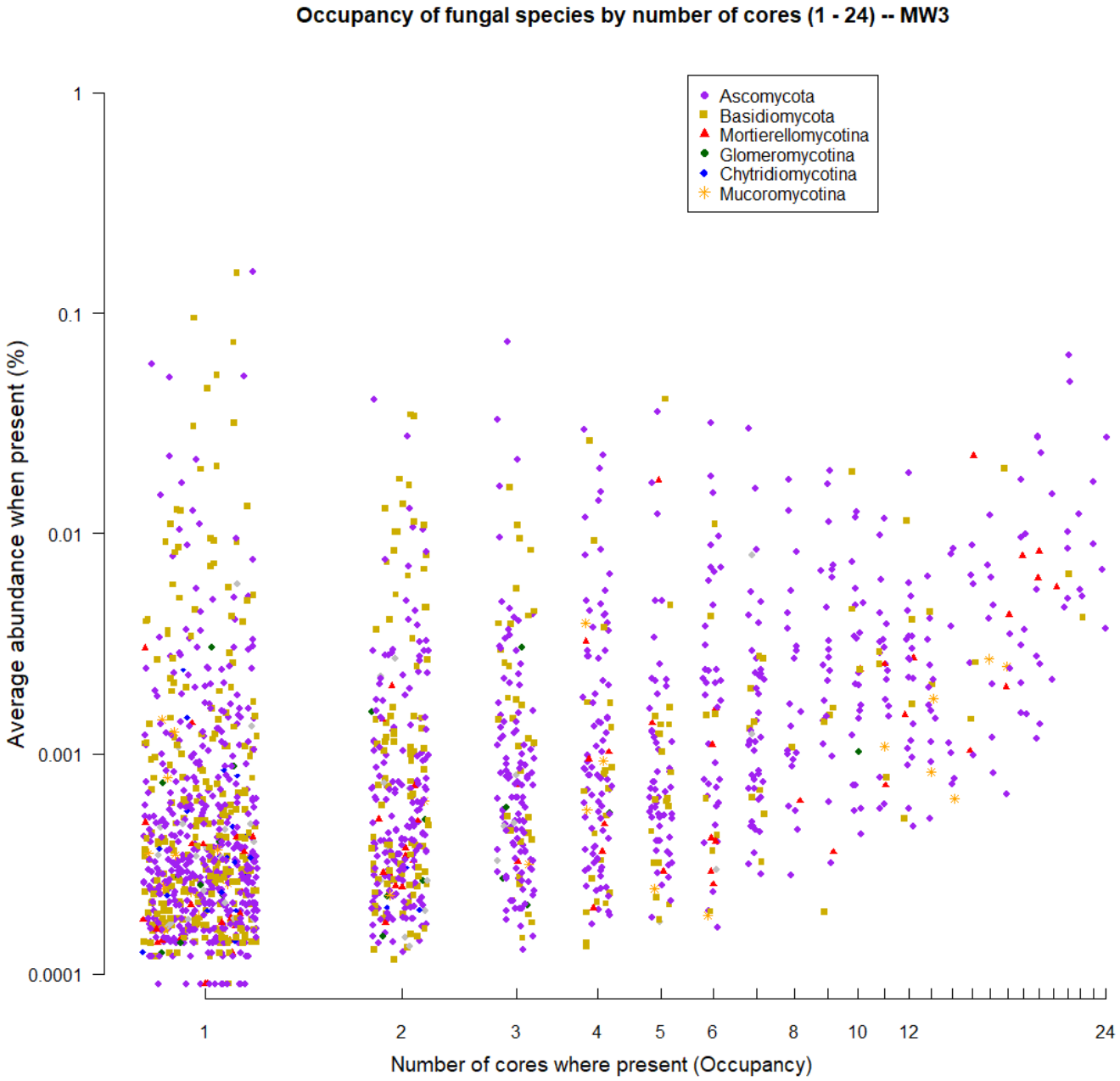
C10. *[Continued]*



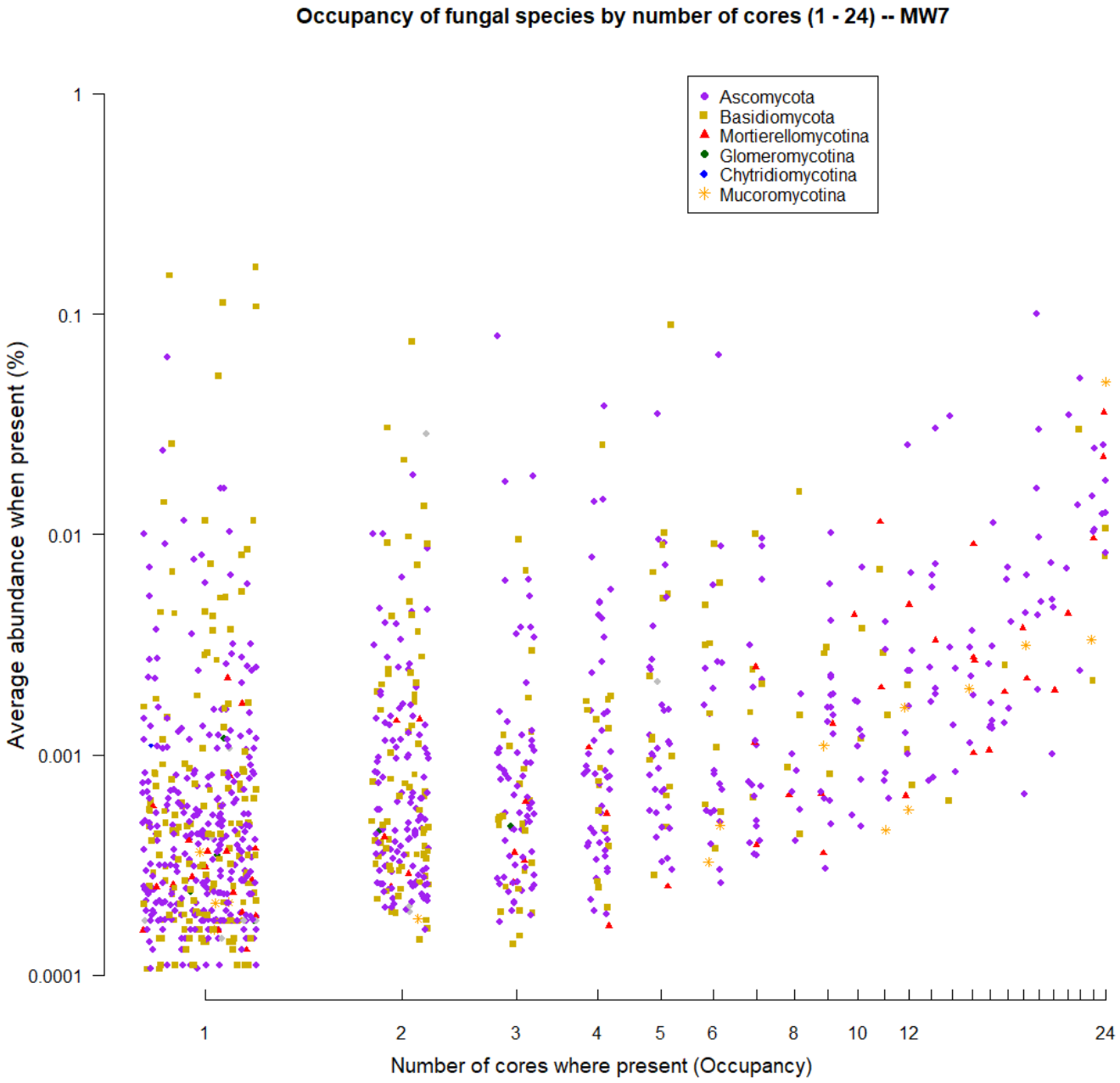
C11. Average % occupancy when present of different fungal species coloured by phylum for 24 cores from each sampling plot.



C11. *[Continued]*



C11. *[Continued]*



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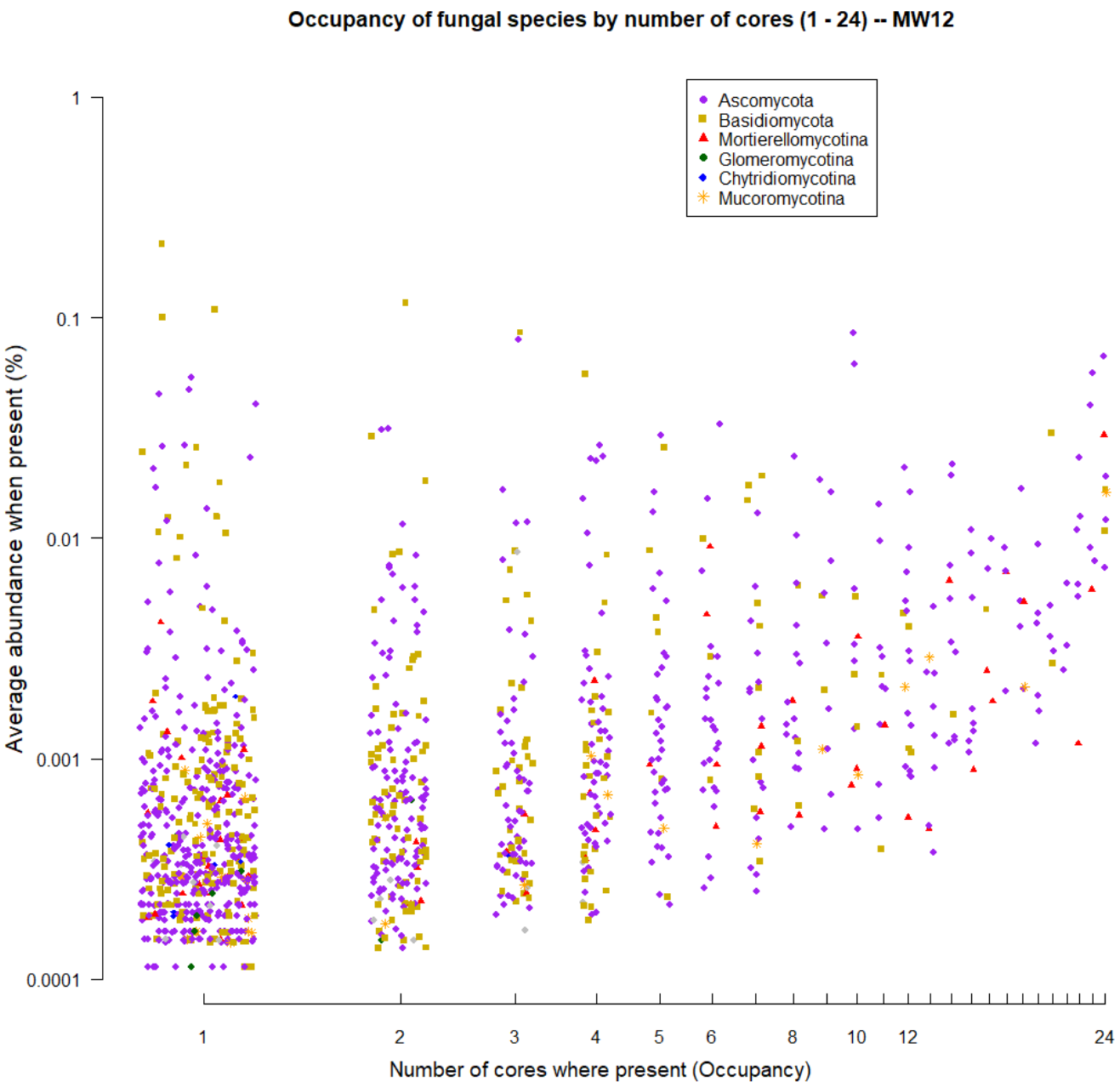
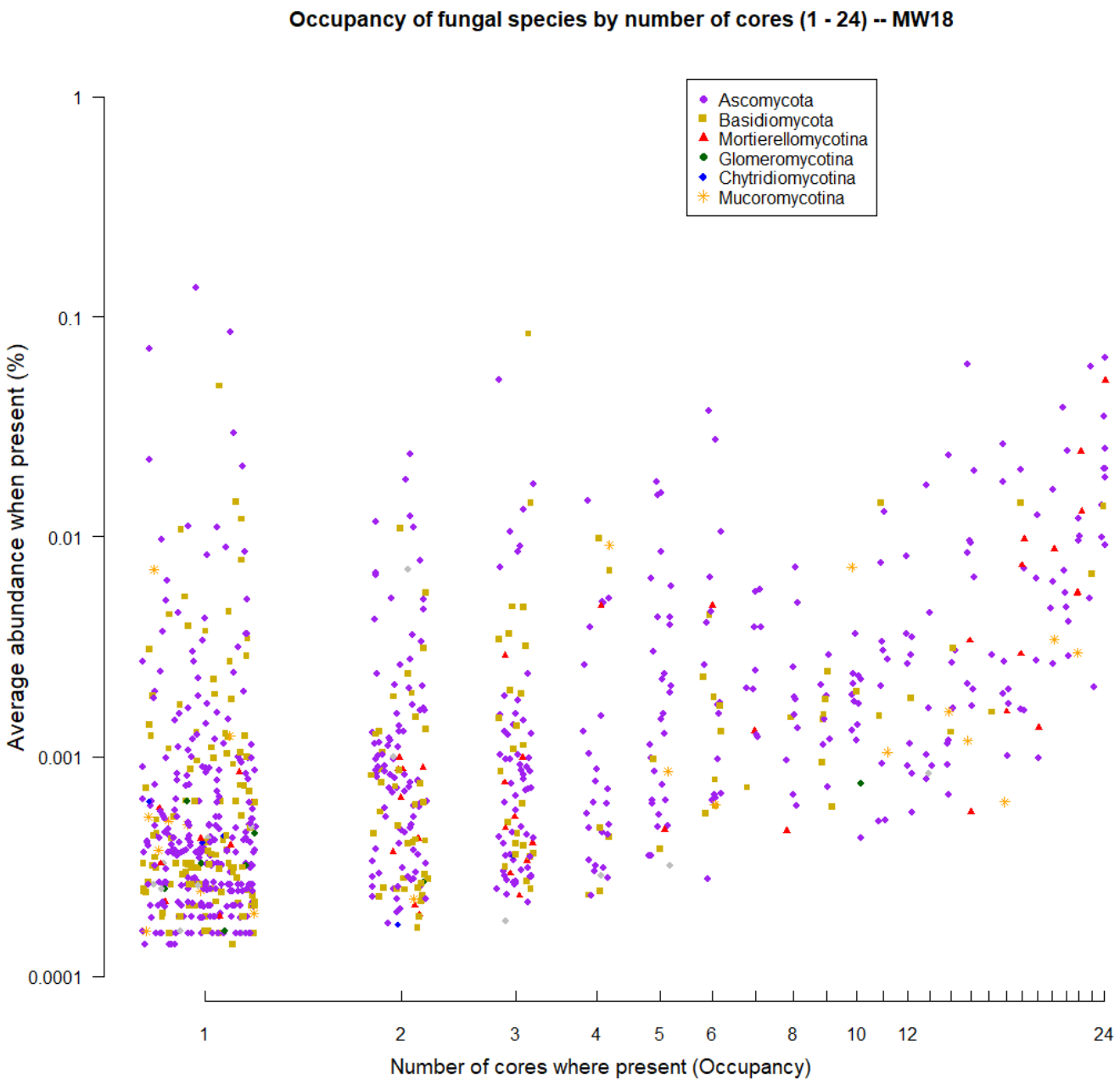
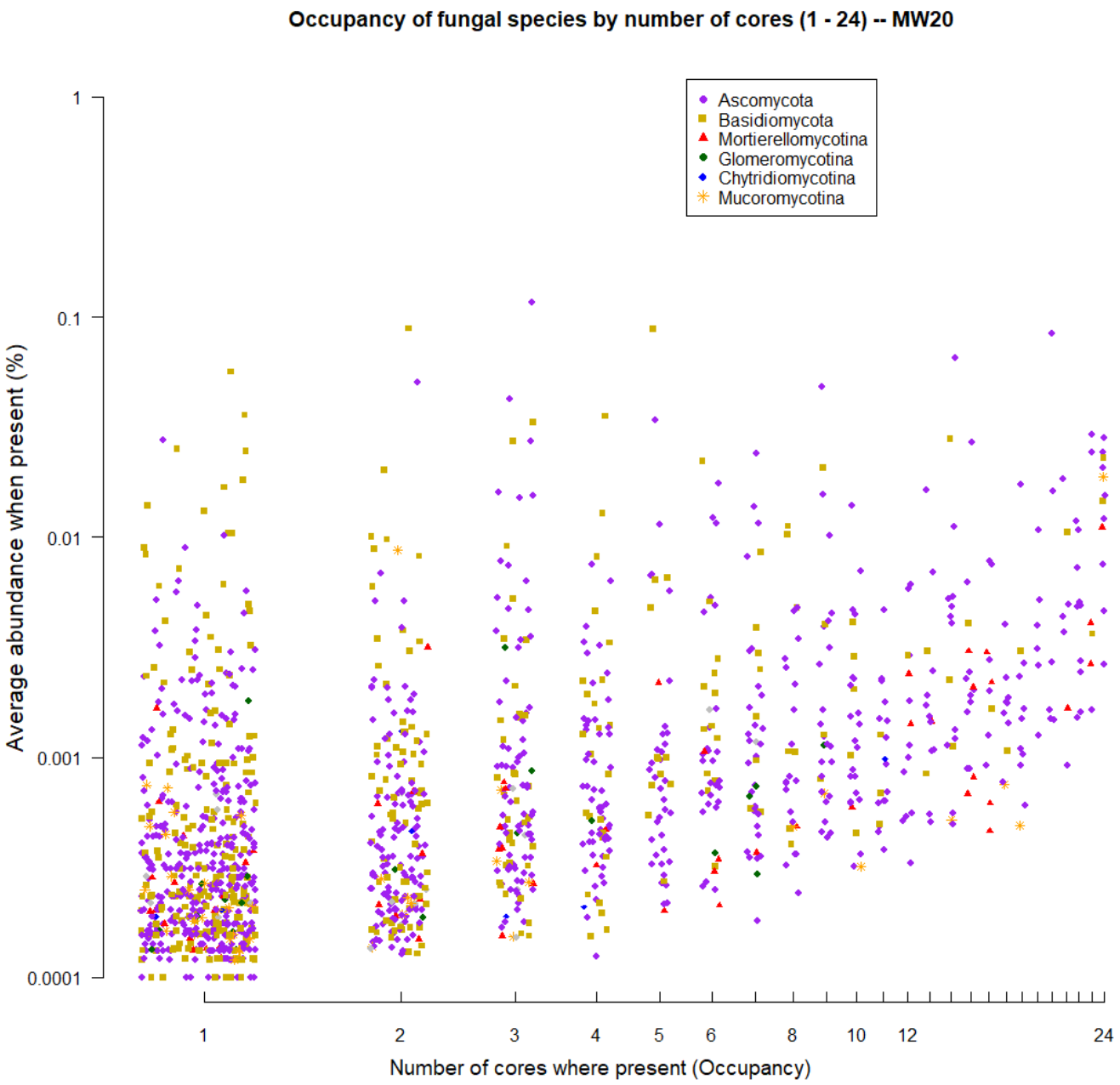


C11. *[Continued]*

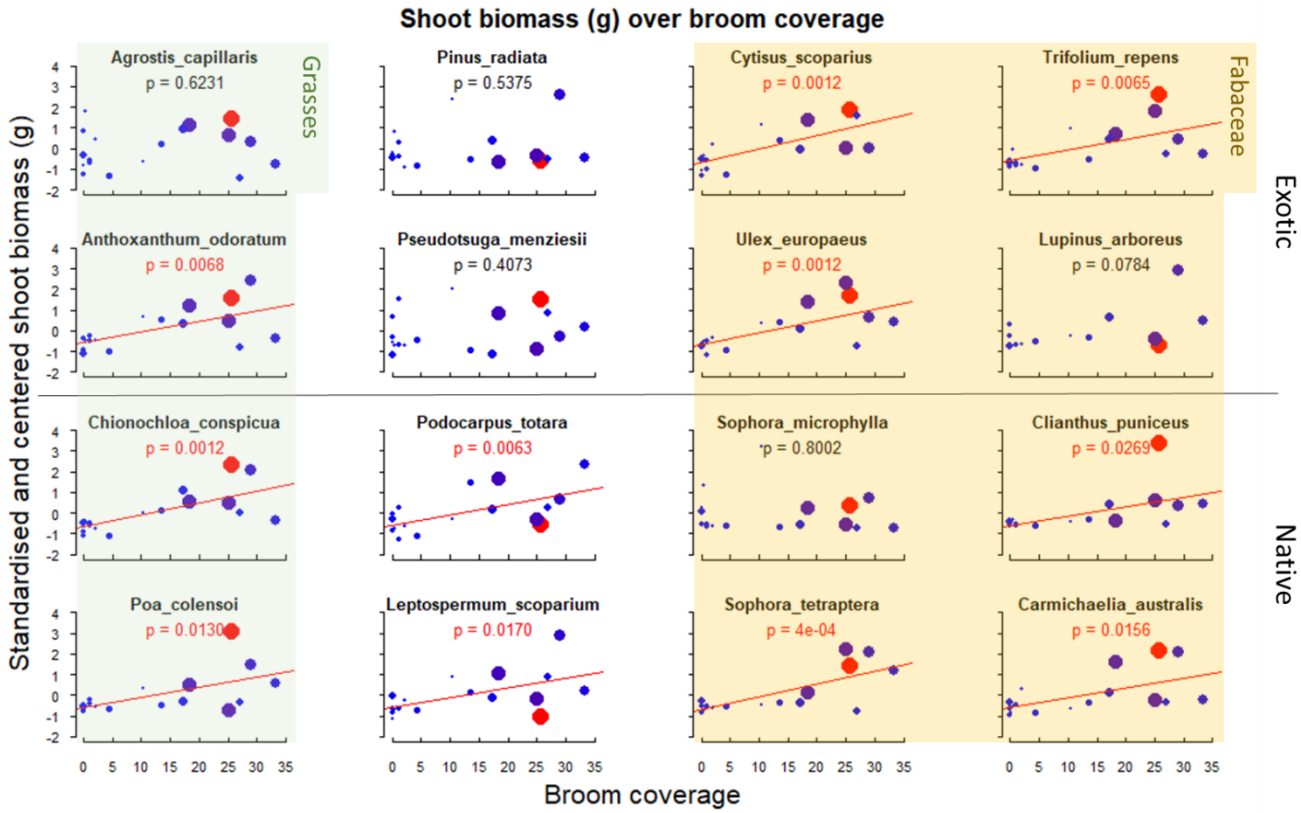


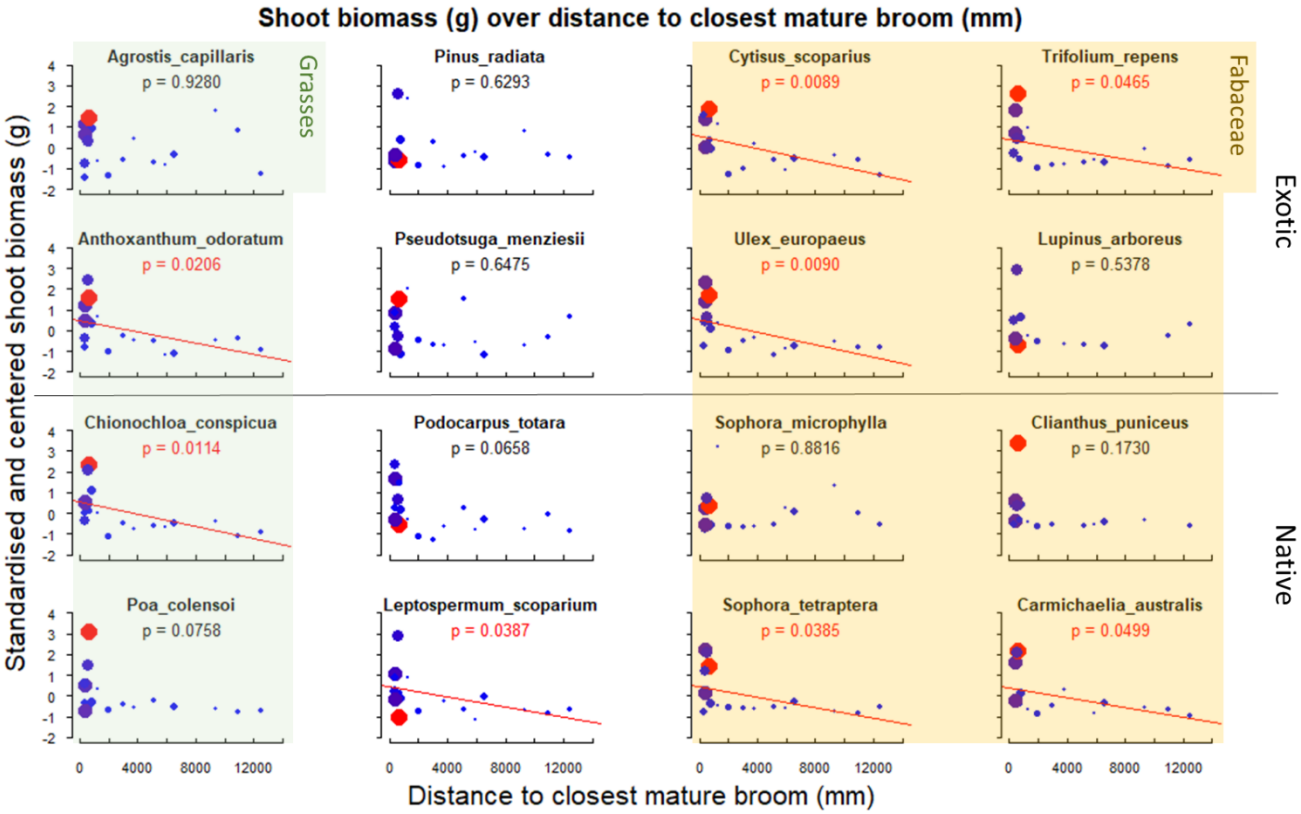
C11. *[Continued]*



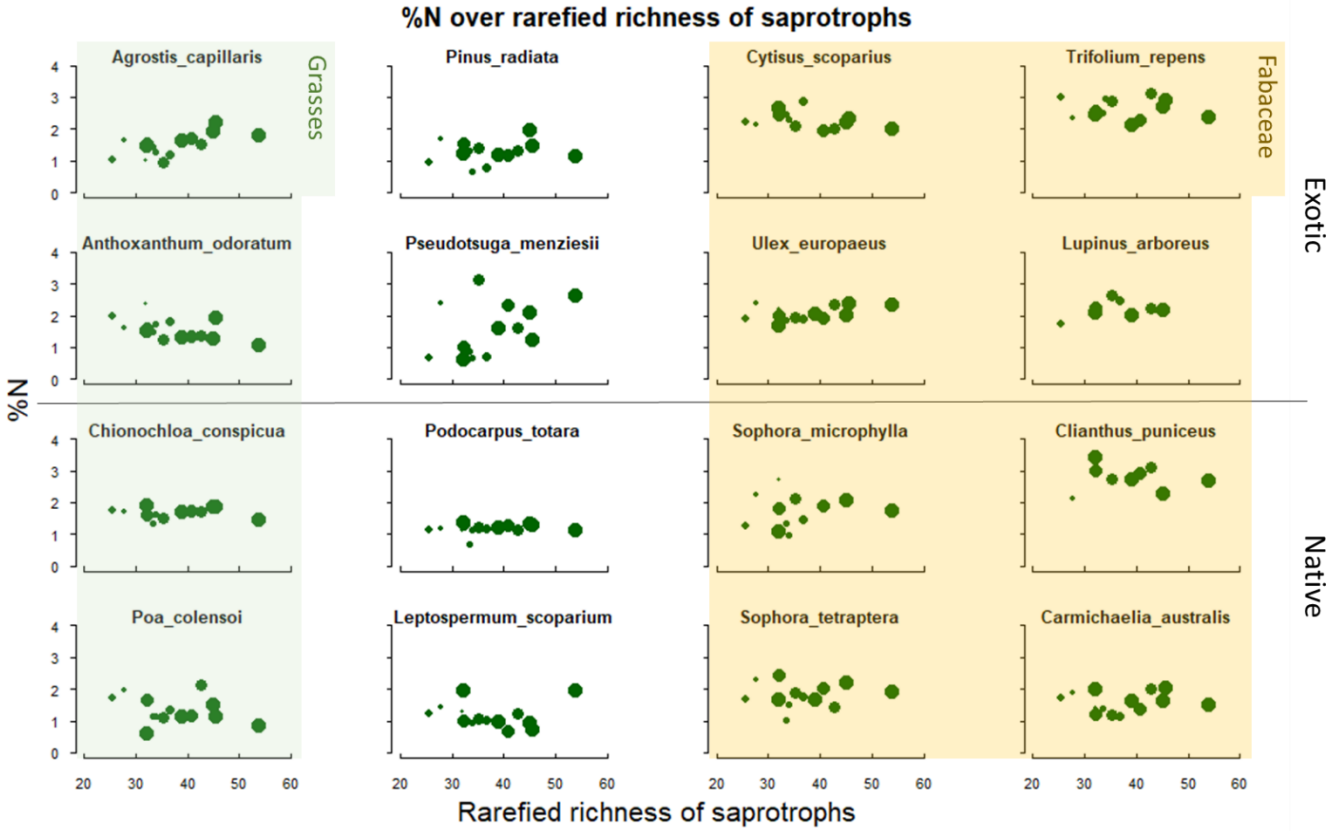
Appendix D (Supplement Chapter 5)

D1. Standardised and centred aboveground dry biomass (g) over *C. scoparius* (i.e., broom) coverage (above) and distance to closest mature *C. scoparius* (below). Size of points is scaled according to the square root of AMF rarefied richness and colour ranges from low AMF proportional abundance (blue) to high AMF proportional abundance (red). Regression lines are shown when $P < 0.05$.

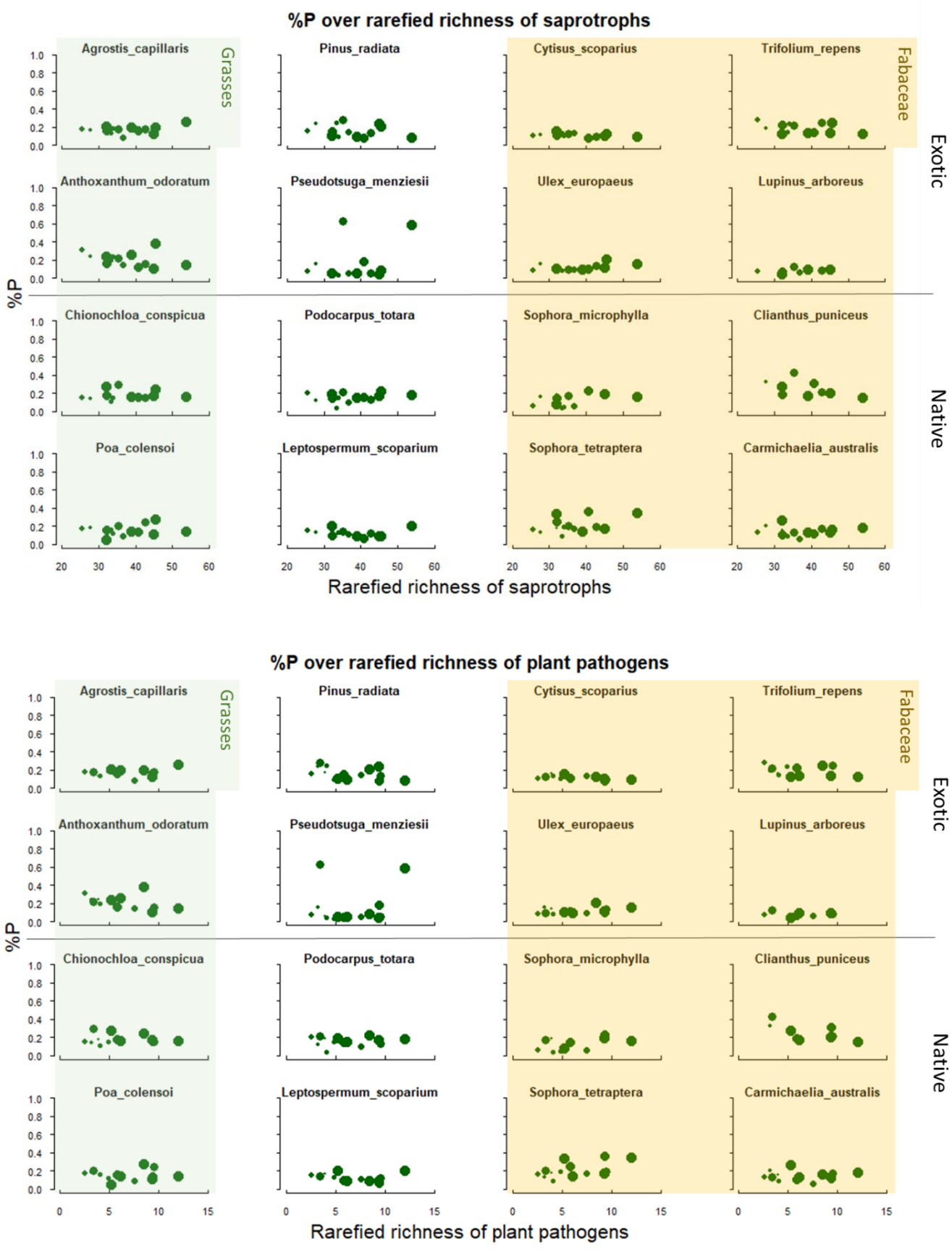




D2. Shoot % N over rarefied richness of saprotrophs (i.e., decomposers). Size of points is scaled according to the square root of *C. scoparius* coverage.



D3. Shoot % P over rarefied richness of saprotrophs (above) and over rarefied richness of plant pathogens (below). Size of points is scaled according to the square root of *C. scoparius* coverage.



Appendix E – Allen *et al.* (2020)

Received: 1 September 2019 | Accepted: 3 May 2020

DOI: 10.1111/1365-2745.13433

Journal of Ecology | BRITISH ECOLOGICAL SOCIETY

RESEARCH ARTICLE

Community-level direct and indirect impacts of an invasive plant favour exotic over native species

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Funding information
Tertiary Education Commission; Royal Society of New Zealand

Handling Editor: James Cahill

Abstract

1. Indirect interactions mediated by shared enemies or mutualists (i.e. apparent competition) can influence whether invasive plants harm or benefit co-occurring species. However, studies to date have largely examined single pairwise interactions, limiting our understanding of the interplay among different types of interactions and whether indirect impacts systematically favour native or exotic species. Predicting indirect interaction strength has also proven challenging, and it remains unclear whether the strengths of different indirect interactions are correlated.
2. We conducted a field experiment in a grassland invaded by Scotch broom *Cytisus scoparius* to compare the strength of its indirect impacts, via both soil fungi and herbivores, on 21 native and exotic legume species growing in pots buried in the ground. Direct interactions of plants with soil fungi were controlled using nylon mesh pot windows of differing porosity (1 or 38 μm) to prevent or allow soil fungi hyphal growth. Arthropod herbivores were controlled through spraying pyrethrum pesticide. To assess indirect impacts, interactions were compared between plants adjacent to or 50 m away from an extensive Scotch broom invasion. We measured plant performance (survival, height and biomass), arthropod and hare herbivory, and rhizobia nodulation.
3. Despite increasing arthropod herbivory of both native and exotic plant species, Scotch broom had a net positive impact on their survival and growth, through sheltering them from abiotic stress, and indirectly via beneficial soil fungi and release from hare browsing. Soil fungi also increased arthropod herbivory, decreased rhizobia nodulation and disproportionately promoted the growth of exotic plants. Overall, exotic plants experienced stronger interactions, which favoured them with beneficial soil fungi and rhizobia but not hare browsing. Finally, indirect interaction strength was not correlated among indirect interactions mediated by different interaction partners.
4. *Synthesis*. We demonstrate that invaders affect their competitors through multiple interacting indirect pathways that were stronger than direct 'nurse plant' effects, emphasizing the importance of a community-level approach to studying biological invasions. Exotic species experienced stronger positive and negative impacts than

Warwick J. Allen and Ralph Wainer contributed equally to this work.

natives, but were facilitated overall, potentially contributing to exotic dominance in communities.

KEYWORDS

apparent competition, *Cytisus scoparius*, indirect facilitation, invasive species, mutualism, nurse plant, plant–fungi interactions, plant–herbivore interactions

1 | INTRODUCTION

Invasive species are exotic species that spread rapidly within a new range (Richardson et al., 2000), often with positive and negative impacts on various aspects of environmental, economic and societal well-being (Pimentel, Zuniga, & Morrison, 2005; Vilà et al., 2011). Many of their impacts occur through direct interactions of invasive species with other species in the community (Tylianakis, Didham, Bascompte, & Wardle, 2008). However, indirect interactions, defined as impacts of one species on the growth, fitness, or population dynamics of another through changes in the population or behaviour of a third species, may be as important as direct interactions in influencing invasion success and impacts (Bhattarai, Meyerson, & Cronin, 2017). Given their disproportionately high biomass, abundance (van Kleunen, Weber, & Fischer, 2010) and generalist associations (Bartomeus, Vilà, & Santamaría, 2008; Moora et al., 2011), invasive species may be expected to engage in strong indirect interactions with the surrounding community. Yet, the relative importance of direct and indirect interactions in modifying invasive species impacts remains unresolved.

Indirect interactions may be mediated by mutualists or enemies. For example, apparent competition describes negative interactions between two or more species mediated by changes in the population or behaviour of shared enemies (Holt, 1977; Holt & Bonsall, 2017). Invasive plants can act as a reservoir for harmful herbivores (Bhattarai et al., 2017; Enge, Nylund, & Pavia, 2013; Orrock, Dutra, Marquis, & Barber, 2015) and pathogens (Borer, Hosseini, Seabloom, & Dobson, 2007; Power & Mitchell, 2004), where exotic and native antagonists can spill over and spill back, respectively, to depress the growth of neighbouring native plants (Allen, Meyerson, Flick, & Cronin, 2018). Conversely, indirect interactions can link species via shared mutualists, leading to several potential outcomes (Dickie, Bufford, et al., 2017). For example, plants indirectly interact with one another via shared fungal mutualists, generating both positive and negative impacts on plant performance via common mycelial networks and altered fungal inoculum potential (Horton, 2015; Newman, 1988; van der Heijden & Horton, 2009). However, much of the literature has focused on the negative indirect impacts of invaders, with studies of indirect facilitation of native species via shared mutualists largely limited to plant-pollinator systems (Bartomeus et al., 2008; Charlebois & Sargent, 2017; but see Dickie et al., 2014), leaving it unclear whether indirect impacts of invasive species on native community members are systematically positive or negative.

Another major limitation of existing studies of indirect impacts is that most have been between invasive and native species (Allen et al., 2018; Bhattarai et al., 2017; Enge et al., 2013), with limited consideration of indirect interactions between exotic species (including those deemed to be invasive). In one of the few published examples, invasive *Pinus contorta* has been shown to facilitate ectomycorrhization of exotic *Pseudotsuga menziesii* (Dickie et al., 2014). Moreover, exotic trees tend to share more ectomycorrhizal fungi than natives (Dickie, Cooper, Bufford, Hulme, & Bates, 2017), suggesting indirect facilitation may be occurring among exotic plants. However, the impacts of these indirect interactions on plant performance in native-exotic systems remain largely unknown. If indirect facilitation occurs between two exotic species via a shared interaction partner, this may represent an indirect pathway to 'invasional meltdown' (Simberloff & Von Holle, 1999), which has received little attention from researchers relative to direct facilitation (Braga, Gómez-Aparicio, Heger, Vitule, & Jeschke, 2018). On the other hand, apparent competition between two exotic species could represent an indirect form of local biotic resistance ('invasional interference'; Yang, Ferrari, & Shea, 2011). Overall, indirect impacts may be expected to be stronger for exotic than native species, due to their generally higher density and biomass (van Kleunen et al., 2010) and tendency to be generalist in their species interactions (Bartomeus et al., 2008; Moora et al., 2011).

Indirect interactions among species are highly context dependent, which adds further complexity and hampers predictability of net indirect interaction strength at the community level. For example, different groups of interaction partners (i.e. herbivores and pollinators) may indirectly influence one another via shared host plants (Franzini, Azcon, Latanze-Mendes, & Aroca, 2010; Koricheva, Gange, & Jones, 2009), and the strength of indirect interactions that they mediate between plants may also be correlated (Fontaine & Thébault, 2015; Sauve, Thébault, Pocock, & Fontaine, 2016) because different organisms can respond similarly to plant cues (Theis, 2006). Thus, investigating pairwise interactions in isolation, or even single guilds of interactions, risks overlooking important indirect interactions among different community members. Indirect impacts of invasions have previously been studied at the community level (e.g. Bumbeer, da Rocha, Bornatowski, de Castro Robert, & Ainsworth, 2018; Feit et al., 2018; O'Dowd, Green, & Lake, 2003), but largely through observation and modelling rather than manipulative experiments. Investigating the balance of these positive and negative indirect interactions could

improve understanding and prediction of invasion success and impacts, yet no studies to date have compared the relative strength of these different types of interaction at a broader community level (i.e. soil biota-plant-herbivore), or their impact across multiple native and exotic taxa.

Here, we experimentally examine the indirect impacts of the global grassland invader Scotch broom *Cytisus scoparius* (L.) Link (Fabaceae) (hereafter broom), on the survival and performance of 10 native and 11 exotic legume species via shared soil fungi and herbivores. By burying potted plants of each species either adjacent to or 50 m away from a large-scale broom invasion and manipulating the presence of indirect pathways via exclusion of soil fungi and arthropod herbivores, we tested the following predictions: (a) Broom impacts the survival and growth of other plant species through indirect interactions mediated by mutualists and enemies; (b) Exotic plant species experience stronger direct and indirect impacts of broom than natives; and (c) Plants that experience strong indirect impacts of broom mediated by herbivores also experience strong indirect impacts mediated by soil fungi.

2 | MATERIALS AND METHODS

2.1 | Study location

The experiment was conducted at Brooksdale Station, Canterbury, New Zealand (−43.3067 N, 171.7673 W, elevation = 647 m). The site features a large (~0.5 km^2) broom invasion bordering a montane grassland that is uninvaded by broom (Supporting Information S1, Figure S1). The uninvaded grassland community (hereafter, we use the term uninvaded to refer to the community where broom is not present as an invader) was dominated by exotic *Agrostis capillaris*, *Festuca rubra*, *Hieracium pilosella* and diverse but sparse native subshrubs (Supporting Information S2, Table S1). This uninvaded community has a history of being grazed by livestock for several weeks in spring each year (but not during our experiment) and, importantly, any broom seedlings are controlled by applying herbicide spray to individual plants every 2–3 years. This manual control of broom means that the invasion front was human-controlled, rather than being driven by any hidden environmental gradients. In the broom-invaded community, broom was not managed and dominated a similar background community of *A. capillaris*, *F. rubra* and *Dactylis glomerata*, but few other plant species (Supporting Information S2, Table S1, Figure S2).

2.2 | Study species

Scotch broom is a perennial, nitrogen-fixing shrub that grows in open and disturbed sites across a wide range of soil types. It is native to western and central Europe but is invasive on all other continents except Antarctica, with impacts on forestry, agriculture and native communities (Jarvis, Fowler, Paynter, & Syrett, 2006; Potter, Kriticos, Wait, & Leriche, 2009). Broom has high biomass and density combined with diverse species interactions (Lafay & Burdon, 2006; Memmott, Fowler, Paynter, Sheppard, & Syrett, 2000), and hence strong potential for indirect impacts. To measure the indirect impacts of broom via shared soil fungi and arthropod herbivores, we used 21 plant species from the same family as broom: Fabaceae (legumes; Table 1). We focused on legumes to minimize phylogenetic distance from broom and thus improve the likelihood of them sharing some interaction partners, and our ability to detect indirect impacts. Many of the experimental species share similar habitat or co-occur with broom in New Zealand; however, co-occurrence was not a requirement for species selection, with species instead chosen to maximize phylogenetic diversity within Fabaceae and based on availability of seed or cuttings.

2.3 | Experimental setup and design

There were four treatments in a fully crossed 2 × 2 × 2 × 2 factorial design with five replicate blocks and 21 plant species (block and species were random effects), resulting in a total of 840 potted plants. The first treatment was plant provenance: the species used for the experiment comprized 10 native and 11 exotic species

TABLE 1 List of native and exotic legume species used in the field experiment

Plant species	Common name	Provenance
Carmichaelia appressa	Prostrate broom	Native
Carmichaelia australis	Common native broom	Native
Carmichaelia stevensonii	Weeping broom	Native
Carmichaelia williamsii	William's broom	Native
Clianthus maximus	Kākābeak	Native
Clianthus puniceus	Kākābeak	Native
Sophora longicarinata	Limestone kōwhai	Native
Sophora microphylla	Small-leaved kōwhai	Native
Sophora prostrata	Prostrate kōwhai	Native
Sophora tetraptera	Large-leaved kōwhai	Native
Cytisus proliferus	Tree lucerne	Exotic
Cytisus multiflorus	Portuguese broom	Exotic
Cytisus scoparius	Scotch broom	Exotic
Genista monspessulana	Montpellier broom	Exotic
Lupinus arboreus	Tree lupin	Exotic
Lupinus polyphyllus	Russell lupin	Exotic
Medicago sativa	Lucerne	Exotic
Spartium junceum	Spanish broom	Exotic
Trifolium pratense	Red clover	Exotic
Trifolium repens	White clover	Exotic
Ulex europaeus	Gorse	Exotic

(including broom; Table 1; Figure 1). The second treatment was designed to manipulate fungal growth between pots and the surrounding soil: pots were fitted with 10 cm × 6 cm nylon mesh windows of differing porosity (1 vs. 38 μm) to prevent or allow fungal hyphae growth, respectively (Figure 1) (Johnson, Leake, & Read, 2001; Teste, Karst, Jones, Simard, & Durall, 2006). The third treatment was designed to exclude or allow impacts of arthropod herbivores: plants were sprayed with pyrethrum pesticide (Yates, Christchurch, New Zealand) or a water control. The fourth treatment was plant location: pots were buried adjacent to the broom invasion or 50 m away in the uninvaded grassland (Figure 1; Figure S1). This treatment enabled comparison of the strength of herbivore or fungal interactions (i.e. the effect of the above three treatments) between invaded and uninvaded communities, which we interpreted as the indirect impact of broom (i.e. apparent competition or indirect facilitation) (see Box 1 for further interpretation details). Pots in the invaded community were situated on the edge of the broom invasion to minimize any shading effects that were not present in the uninvaded grassland. A randomized blocked design was used to account for any possible biotic or abiotic gradients along the transects, although we observed no systematic changes in the dominant plant community in preliminary surveys (Supporting Information S2).

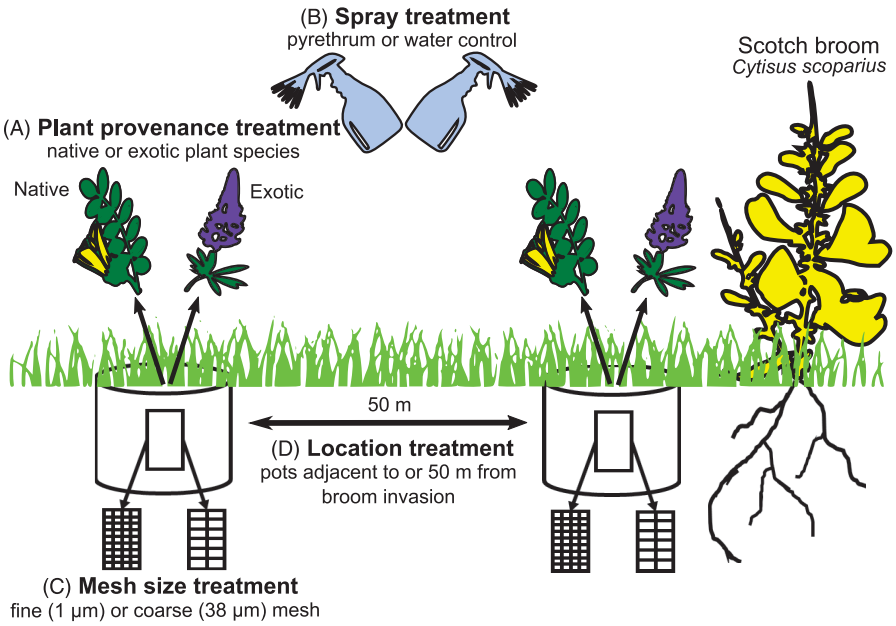

FIGURE 1 Summary of experimental design, illustrating the fully crossed (A) plant provenance treatment (native or exotic plant species); (B) spray treatment (pyrethrum pesticide spray to exclude arthropod herbivores or water control); (C) mesh size treatment (fungal hyphae can grow in and out of pots with 38 μm mesh, whereas 1 μm mesh blocks their growth); and (D) pot location treatment (adjacent to or 50 m away from Scotch broom *Cytisus scoparius*)

BOX 1 Interpretation of treatment main effects and interactions in mixed model analyses

Main effects of location, mesh size or spray treatment with no interactions were interpreted as direct impacts of broom, soil fungi and arthropod herbivores, respectively. However, the location treatment main effect could also be driven by unmeasured indirect impacts of broom, potentially mediated by other taxa that we did not manipulate or quantify such as fungal endophytes or changes in the surrounding plant community. We interpreted the mesh size treatment as a fungal effect (meaning that fungi from outside the pot colonized the soil and experimental plant, fungi from inside the pot grew into the surrounding environment, or both), yet there are other possible interpretations that cannot be entirely ruled out, such as variable water drainage or movement of nutrients or other resources across the different mesh sizes. However, we may expect these abiotic impacts to be consistent in the invaded and uninvaded community and between native and exotic species, which was not supported by the results.

Critically, we interpreted pairwise interactions of plant location with exclusion treatments (i.e. mesh or spray effects differ between the invaded and uninvaded community) as indirect impacts of broom, an approach that has been previously used to assess indirect impacts of invasive plants (Bhattarai et al., 2017). Similarly, because hare browsing, arthropod herbivore damage and rhizobia nodulation variables were not treatments themselves, we interpreted main effects of plant location for these variables as an indirect impact of broom mediated by changes in the density or behaviour of these taxa. We contend that broom is the major difference between the invaded and uninvaded plant communities (Supporting Information S2) and thus the main contributor to the differences in interactions observed between plants in the two locations. Effects involving plant provenance were interpreted as differences in inherent growth (main effect of provenance for plant performance variables), direct interactions (provenance × location/mesh/spray treatments) and indirect interactions (location × provenance × mesh/spray treatments) between native and exotic plants. Finally, interactions between the mesh and spray treatments were interpreted as host plant-mediated indirect interactions between soil fungi and arthropod herbivores.

Plants were grown from seed (obtained from field populations, New Zealand Tree Seeds, and Prebble Seeds, Christchurch, New Zealand), except *Carmichaelia appressa* and *C. australis*, which were propagated from stem cuttings treated with Clonex root hormone gel (Yates, Auckland, New Zealand). Following germination, seedlings were transplanted into pots on 16–17 November 2017 and maintained under regularly watered greenhouse conditions for 4 weeks. Pots were 1.5 L polyethylene terephthalate bottles (Alto, Christchurch, New Zealand) with tops removed and a 2.5 cm layer of 5–10 mm diameter pebbles (Intelligro, Christchurch, New Zealand) was added to the bottom of the pots to improve drainage. Soil was mixed 50:50 with sand and was collected from the top 30 cm of the uninvaded grassland at the field site to represent live soil naïve to invasion and without any nitrogen increase associated with broom (Broadbent et al., 2017). Potted plants were established in the field between 13 and 23 December 2017, by fitting pots into 20 cm deep holes drilled with a 10 cm diameter earth auger, ~1 m apart in a transect (Figure S1). Plant survival was assessed after two weeks and dead seedlings replaced. Seedlings were watered twice (January–February 2018) or once (March–April) per week and weeded as necessary.

2.4 | Data collection

To assess initial growth, the height of all plants was recorded six weeks after transplanting into the field (5 February 2018), and was measured again at harvest. Surviving plants (n = 570) were harvested between 16 and 30 April 2018. Roots were washed, and above- and belowground biomass separated into paper bags and dried for 72 hr at 55°C before being weighed. Using ImageJ software (Rasband, 2018), we quantified cumulative arthropod herbivore percent leaf damage from photographs of all leaves from each plant (excluding plants browsed by hares), estimating the undamaged leaf area by extrapolation. Finally, the number of root nodules was counted for each plant as a proxy for plant–rhizobia interactions and standardized by root dry mass (nodules per gram of root dry mass) to remove confounding effects of the experiment treatments on plant size, which may be correlated with the number of nodules.

A few weeks into the experiment, we began finding plants that were browsed down to near the base of the stem. Based on observations at the study site and the characteristic damage, we determined that European hares *Lepus europaeus* were responsible. Rather than attempting to control the hares, we used their herbivory as an opportunity to separately assess the indirect impact of broom mediated by hares. Because it was impossible to determine how much plant tissue had been browsed, hare herbivory was quantified as present or absent. Hare-browsed plants that still had healthy roots or live aboveground tissue were not considered dead in the survival assessment. Because hare herbivory was not a controlled treatment, we excluded hare-browsed plants from analyses of plant height, aboveground biomass, total biomass and arthropod herbivore damage, and included hare herbivory as a covariate for analyses of belowground biomass and rhizobia nodulation (see below and Supporting Information S3 and S4 for details).

2.5 | Data analysis

We used model selection based on the Akaike Information Criterion corrected for small sample size (AICc) to assess strength of direct and indirect interactions in this montane grassland community, selecting the best-fitting mixed-effect models from a set of candidate models for each response variable (Burnham & Anderson, 2010). The full model for all response variables included plant provenance, pot location, mesh size, spray treatment and all possible interactions as fixed effects (15 total variables). Hare browsing was included as a fixed covariate for analyses of belowground biomass and nodulation. Random intercepts were included in all models for plant species and experimental block, to account for among-species variation and possible environmental gradients, respectively. Candidate models were based on subsets of the full model, using all possible combinations of the explanatory variables, but with the restrictions that main effects must also be present in models containing interactions, and that random effects were retained in every model combination to account for structural aspects of our design. We ranked candidate models from lowest to highest AICc and models with ΔAICc (=$AICc_i - AICc_{min}$) of two or less were deemed to have substantial support (Burnham & Anderson, 2010). Each influential variable was then subjected to post-hoc Tukey tests (Bonferroni corrected where appropriate). Different model error distributions were used depending on the response variable, and some variables were transformed to meet model assumptions (Supporting Information S3, Table S2 and Supporting Information S4 for further analysis details). Although some plant performance variables were correlated (Supporting Information S5, Table S3), we report results for all because they represent different components of plant growth that are of interest to researchers and that were expected to vary in their responses to different treatments. The total number of plants included in each analysis also varied depending on the response variable being investigated, number of plants alive and the presence of hare browsing (Supporting Information S3, Table S2). Nodulation was assessed using a two-stage model, where we first examined treatments that were influential to the presence or absence of nodules, and then the number of nodules per gram of root biomass for plants with nodules. To make effects comparable across different response variables, we calculated Cohen's d effect size (= model coefficient/standard deviation) for contrasts that were significant in post-hoc Tukey tests. The focus of the main text is on comparing the size of these effects, but in the interest of transparency we present the full results and interpretation of the model selection and post-hoc analyses in Supporting Information S6 and S7. Furthermore, to examine whether plant performance was influenced by our observed direct interactions (i.e. hare herbivory, arthropod damage and nodulation), we ran separate linear mixed models using total biomass as the response variable, the observed interaction as

the explanatory variable, and plant species and experimental block as random effects.

Finally, we considered but elected not to use structural equation modelling to analyse the data, because incomplete observations for some response variables (e.g. arthropod herbivory, harvest height, aboveground biomass and total biomass could not be assessed for hare-browsed plants) meant that a comprehensive SEM containing all response variables was not possible, and any reduced analysis would result in a significant loss of data and inference power for hypothesis tests of all pathways.

We also extracted species-level direct and indirect impacts of broom from the analyses to test whether (a) direct or indirect impacts of broom were stronger; and (b) indirect impacts mediated by herbivores and soil fungi were correlated. To address these questions, we estimated species-level coefficients by including random slopes for each experimental plant species in best-fitting models where direct (i.e. location main effects for plant performance variables) or indirect (i.e. a location × mesh or location × spray interaction for plant performance variables or a location main effect for observed arthropod herbivore damage, hare herbivory and nodulation) impacts of broom were identified. Using these species-level coefficients, we first tested for differences in the strength of direct and indirect interactions using a linear mixed model, with interaction type (direct vs. indirect) as the sole fixed effect and plant species and dependent variable identity (e.g. total biomass, survival, arthropod herbivory) as random effects in the model. Second, we used linear regression to ask whether plants that experienced strong indirect impacts of broom mediated by herbivores also experienced strong indirect impacts mediated by soil fungi. We also tested whether indirect impacts mediated by arthropod herbivores and hares were correlated. Finally, we also tested whether phylogenetic relatedness predicted the strength of indirect interactions (i.e. species-level random effect coefficients). However, because of the limited breadth (i.e. within a single family, Fabaceae) and resolution (i.e. only to genus level) of our phylogeny, we elected to present these results only in Supporting Information S8. Absolute values of interaction strength (i.e. random slope coefficients) were used throughout these analyses because we were interested in the effect magnitude rather than direction, and indirect interaction strength could be both positively and negatively influenced depending on the context (e.g. beneficial indirect facilitation vs. harmful mutualist competition). All analyses were performed in R 3.6.0. (R Development Core Team, 2019) using the LME4 (Bates et al., 2019), MUMIN (Bartoń, 2019), and EMMEANS (Lenth, Singmann, Love, Buerkner, & Herve, 2019) packages.

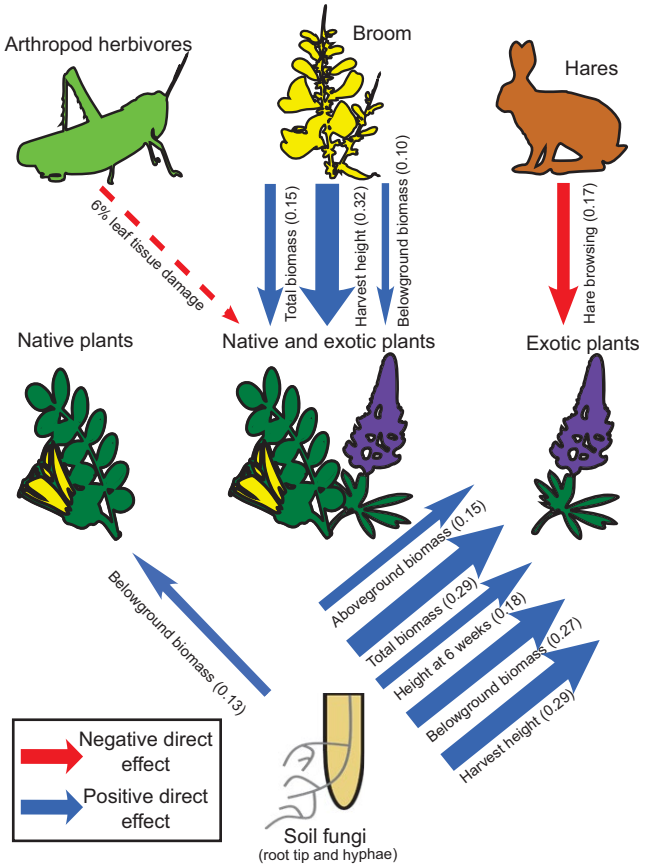

FIGURE 2 Summary of all measured direct effects of Scotch broom *Cytisus scoparius*, herbivores, soil fungi and rhizobia on native and exotic plant species when grown adjacent to broom. Arrows indicate influential main effects retained in best-fitting models and their colour reflects positive (blue) or negative (red) direct impacts on plants from the respective interaction partner. Arrow width represents Cohen's *d* effect sizes (i.e. direct interaction strength, quantified in parentheses) calculated from regression coefficients for the main effect of location (broom), mesh size (soil fungi) and plant provenance (hare browsing and rhizobia nodule presence). The dotted arrow from arthropod herbivores indicates that leaf damage was observed but that the spray treatment did not influence plant performance, and thus a Cohen's *d* effect size could not be calculated

3 | RESULTS

3.1 | Most direct interactions were stronger for exotic than native plants

Plant performance was influenced by many direct (Figure 2) and indirect interactions (Figure 3). Detailed results and interpretation of model selection and post-hoc analyses, including test statistics and *p*-values, are reported in Supporting Information S6 and S7. There was a main effect of location (with no interactions) for several variables, suggesting that broom had a positive direct impact (i.e. likely not via above- or belowground interaction partners, although this cannot be entirely excluded; see Box 1) on total biomass (35% increase), belowground biomass (20% increase) and height at harvest (40% increase) of both native and exotic experimental plant species (Cohen's *d* = 0.15, 0.10 and 0.32, respectively; Figure 2; Figure S5).

There was an interaction between mesh size and plant provenance, indicating that soil fungi had a direct positive impact on height after 6 weeks (54% increase), harvest height (53%), total biomass (112%) and aboveground biomass (111%) for exotic but not native plants (Cohen's *d* = 0.18, 0.29, 0.29 and 0.15, respectively;

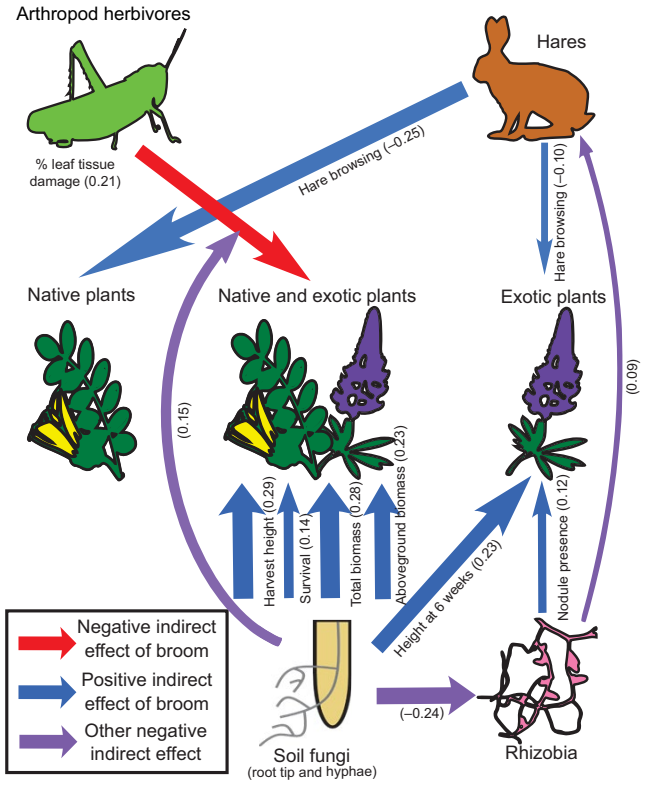

FIGURE 3 Indirect impacts of broom on native and exotic plant species via their shared interaction partners, as determined by the difference in strength of the interaction partner effect between locations (invaded, uninvaded) in the field experiment. Arrows indicate influential effects retained in best-fitting models. Arrow colour reflects whether broom has a positive (blue) or negative (red) indirect impact on the plant, mediated via the corresponding interaction partner. Purple arrows represent negative consequences that have been amplified via indirect impacts of soil fungi or rhizobia on other interaction partners, mediated by the experimental plant. Arrow width represents Cohen's *d* effect sizes (i.e. indirect interaction strength, quantified in parentheses) calculated from regression coefficients and shows the magnitude of the effect of broom (i.e. location) on the interaction

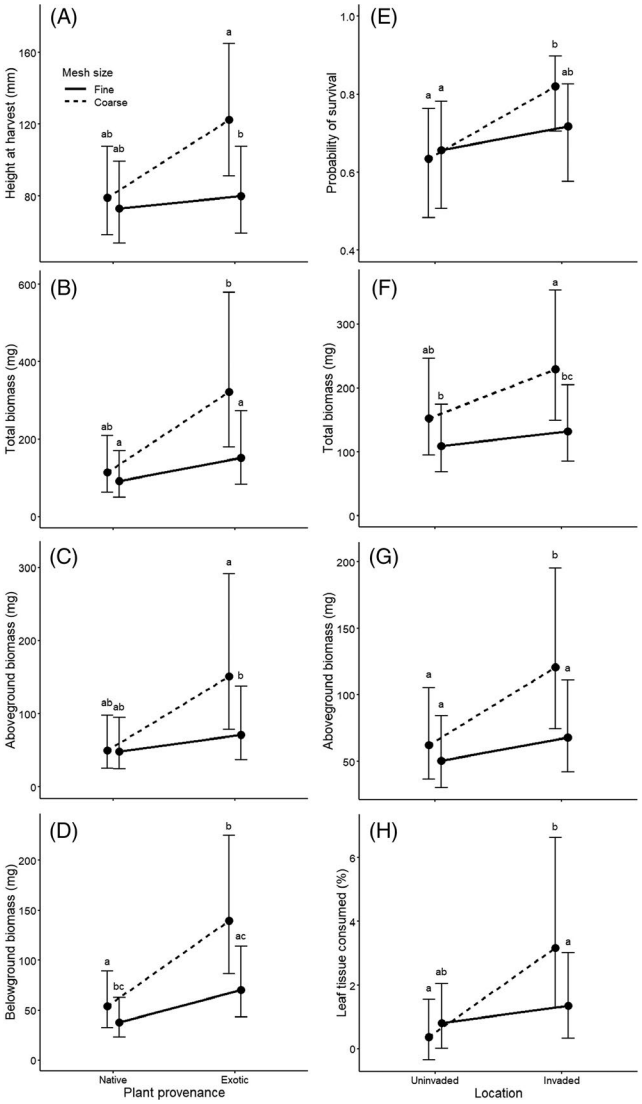

FIGURE 4 Impact of fine and coarse pot mesh on height at harvest (estimated marginal mean ± 95% CI) (A), total biomass (B), aboveground biomass (C) and belowground biomass (D) for native and exotic plant species and on the probability of plant survival (E), total biomass (F), aboveground biomass (G) and leaf tissue damaged by arthropod herbivores (H) in habitat invaded or uninvaded by Scotch broom *Cytisus scoparius*. Different lowercase letters indicate significant differences among means for each variable in post-hoc Tukey tests ($p < 0.05$). The interactions of mesh size with location and plant provenance were identified as influential using mixed-effects model selection (Table S4)

Figure 4A–C, Figures S4 and S6). Soil fungi also increased belowground biomass of native plants, but the effect was twice as strong for exotics (41% and 98% increase, respectively; Cohen's *d* = 0.13 and 0.27, Figure 4D). Furthermore, the number of rhizobia nodules per gram of root biomass was 70% higher for exotic than native plants, although this effect was not strong enough to be significant (Figure S11C).

Aboveground, 44% of plants were browsed by hares. Broom invasion decreased the probability of hare herbivory by 39% and 81% for exotic and native species, respectively, resulting in three times higher hare browsing of exotic than native plants in the invaded community (Cohen's *d* = 0.17; Figure 5). The pyrethrum spray treatment increased hare herbivory of plants in the uninvaded habitat (46% higher), native plants (105% higher) and those grown in pots with fine mesh (87% higher; Cohen's *d* = 0.16, 0.14 and 0.16, respectively; Figure S9). Plants browsed by hares on average had 15% more nodules than undamaged plants (Cohen's *d* = 0.09; Figure S11B) and 38% less biomass (Figure S7). In contrast to hare browsing, arthropod herbivory was low, averaging just 6.3 ± 1.1% of leaf area damaged, did not differ between native and exotic plants, and was unrelated to total biomass. Few herbivores or evidence of their feeding was observed other than several species of Orthoptera, minor gastropod damage, one leafroller caterpillar (Lepidoptera: Tortricidae), and the distinctive leaf-notching damage of clover root weevil (*Sitona obsoletus*, Coleoptera: Curculionidae) on foliage of three *Trifolium* individuals. The pyrethrum spray treatment had no direct impact on any of

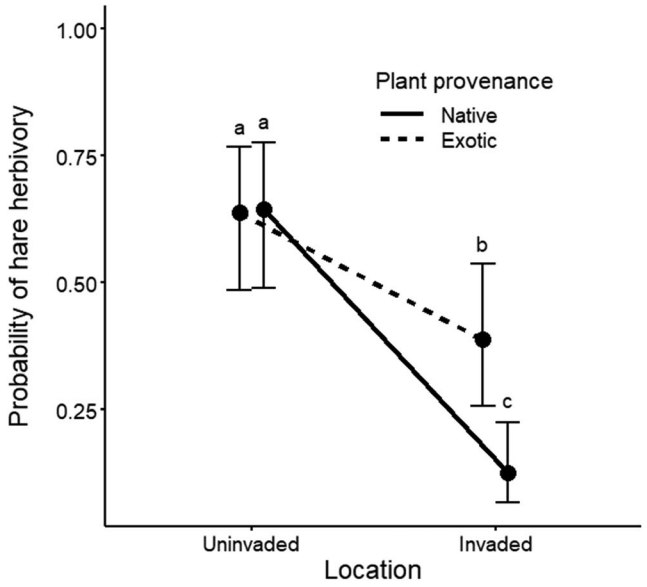

FIGURE 5 The probability of European hare *Lepus europaeus* browsing (estimated marginal mean ± 95% CI) for native and exotic plants in communities invaded and uninvaded by Scotch broom *Cytisus scoparius*. Different lowercase letters indicate significant differences among means in post-hoc Tukey tests ($p < 0.05$). The interaction between plant provenance and location was identified as influential using mixed-effect model selection (Table S4)

the plant performance variables, but reduced herbivore damage by 26%, although this effect was non-significant in post-hoc analyses (Figure S8).

3.2 | Indirect interactions were mostly positive and favoured exotic plants belowground and native plants aboveground

Aboveground, broom invasion had a positive indirect impact on other plants through decreased hare herbivory relative to the uninvaded community, which was stronger for native (81% decrease) than exotic plants (39%) (Cohen's $d = -0.25$ and -0.10, respectively; Figures 3 and 5). Conversely, broom invasion increased arthropod herbivore damage by eightfold relative to the uninvaded community, but only for plants allowed to interact with external fungi via coarse mesh (Cohen's $d = 0.21$; Figures 3 and 4H). The indirect impact of broom on plant height after 6 weeks via belowground interaction partners was positive (54% increase) for exotic plants (Cohen's $d = 0.23$; Figure S4) but no effect was observed for native plants. However, the indirect impact of broom via belowground interaction partners on survival, total biomass, aboveground biomass and height at harvest was positive for both native and exotic plants (Cohen's $d = 0.14$, 0.28, 0.23 and 0.29 respectively; Figure 4E–G; Figure S6). In addition, soil fungi associated with broom increased arthropod herbivore damage by 133% (Cohen's $d = 0.15$; Figure 4H). Finally, the probability of rhizobia nodule presence on roots was 0.99 ± 0.0001 (mean ± *SE*) for exotic plants compared to 0.78 ± 0.001 for natives (Cohen's $d = 0.12$; Figure S10), but this difference was only statistically significant when adjacent to broom or sprayed with the water control. Nodule abundance was also 40% higher when soil fungi were excluded via the fine mesh treatment (Cohen's $d = -0.24$; Figure S11A). Overall, the indirect impacts of broom were over six times stronger than the direct impacts that we measured at the species level ($F_{1,206} = 66.83$, $p < 0.001$). Finally, we found no correlation between the indirect impacts of broom on each plant species that were mediated by herbivores and soil fungi (Supporting Information S8, Table S5).

4 | DISCUSSION

Our field experiment demonstrates how invader impacts occur through many direct and indirect pathways above- and belowground, with stronger positive and negative impacts on other exotic species than natives (summarized in Figures 2 and 3). We found that despite increasing arthropod herbivory, broom caused a net increase in survival and growth of other plant species, both directly, likely through shelter from abiotic stress, and indirectly, via beneficial soil fungi and release from hare browsing (Figures 4 and 5). Soil fungi associated with broom also had negative indirect impacts through increased arthropod herbivory (Figure 4H) and decreased rhizobia nodulation (Figure S11). Overall, the direct and indirect impacts we observed were mostly positive and favoured exotic plants belowground (via soil fungi and rhizobia) and native plants aboveground (via herbivores). Moreover, the indirect impacts of broom that we measured were six times stronger than direct impacts, highlighting the importance of both direct and indirect interactions in driving invasion impacts. Finally, we found little evidence that the strength of indirect interactions can be predicted by the strength of other indirect interactions (Table S5), indicating that a more nuanced approach may be required for future studies.

Survival and growth of other plant species were higher in the invaded community, supporting the concept of 'nurse plants' that facilitates the growth of other species (e.g. Burrows, Cieraad, & Head, 2015; Pugnaire et al., 1996). We extend this concept by identifying potential mechanisms: (a) direct impacts (Figure 2), possibly through shelter from abiotic stress (Carter, Slesak, Harrington, Peter, & D'Amato, 2019), and (b) indirect impacts, mediated by beneficial soil fungi, rhizobia and escape from hare browsing (Figure 3). The lower hare browsing in the invaded community (Figure 5) was likely behaviourally mediated, due to decreased need for predator vigilance and thereby better browsing in open areas (Marboutin & Aebischer, 1996), or the denser broom and grass vegetation limiting hares' ability to visually locate plants. Our result showing facilitative effects of broom differs from studies that have demonstrated negative impacts of invasive plants on native plants through apparent competition mediated by both mammalian and arthropod herbivores (e.g. Bhattarai et al., 2017; Enge et al., 2013; Orrock et al., 2015). However, broom also increased arthropod herbivory eightfold (Figures 3 and 4H), although total leaf damage remained low and was not correlated with plant fitness. These contrasting results could be

because arthropod herbivory measured cumulative damage from the entire community, including both generalists and specialists, whereas hare herbivory was from one generalist that browsed all 21 species. Regardless, these differences highlight how investigating single interactions and native-invasive species pairs precludes making broad conclusions about invader impacts. Here, we present invasive species impacts in a broader community context by investigating several interactions involving 21 species of native and exotic plants, finding that broom impacts were generally consistent within plant provenances rather than the result of idiosyncratic species pairing.

While aboveground indirect impacts of broom may have been mediated by hare behaviour, the positive belowground indirect impacts on neighbours (Figures 3 and 4) were likely a consequence of broom increasing the quantity and/or quality of beneficial soil biota (e.g. mycorrhizal fungi and rhizobia bacteria; Horton, 2015; Newman, 1988). Although the outcome of mycorrhiza-mediated interactions between mature plants and seedlings is most frequently facilitative (48% of studies, van der Heijden & Horton, 2009), negative interactions such as mycorrhiza-mediated growth depression in seedlings competing with a stronger host (Waller, Callaway, Klironomos, Ortega, & Maron, 2016) can also occur (25% of studies), as well as no impact whatsoever (27%). We also cannot rule out the possibility that fungal-mediated impacts could occur via direct and indirect impacts of broom on other plants in the community and their fungal interaction partners (i.e. the broader plant-mycorrhizal network), which then fed back onto our experimental seedlings. Furthermore, soil fungi also had their own negative indirect impacts via increasing arthropod herbivory in the broom-invaded community (Figures 3 and 4H), likely driven by increased plant nutritional value or mycorrhizal suppression of plant defences (Koricheva et al., 2009). Plants that interacted with soil fungi also had lower presence of rhizobia nodules (Figure 3; Figure S11), possibly due to competition between rhizobia and mycorrhiza for limited carbohydrates of the young seedlings (Franzini et al., 2010).

Despite the complexity of interactions described above, some consistent patterns emerged. For example, the direct and indirect impacts we measured were mostly positive and favoured exotic plants belowground and native plants aboveground, with a net advantage to exotic plants. Belowground, exotic plants experienced stronger positive interactions with soil fungi and rhizobia, potentially through greater generality and promiscuity with interaction partners (Moora et al., 2011) or being better hosts (Lekberg, Gibbons, Rosendahl, & Ramsey, 2013). The stronger indirect facilitation of exotic plants by broom via soil fungi and rhizobia could represent an indirect mechanism of invasional meltdown (Simberloff & Von Holle, 1999), potentially leading to ecosystems that are increasingly dominated by exotic species. A meta-analysis by Kuebbing and Nuñez (2016) found similar evidence for competitive interactions, showing that negative competitive impacts of exotic plants on native plants are two times stronger than their impacts on other exotic plants, further suggesting that indirect interactions among multiple native and non-native species could favour exotic dominance of plant communities. A recent global analysis by Stotz et al. (2020) supports this hypothesized outcome, finding that herbaceous grassland species tended to associate with more exotic plant species in their introduced range and with more native plant species in their native range, a pattern that could result from indirect impacts of invasive plants that favour other exotic species over natives.

Aboveground, our findings of greater hare browsing on exotic than native plants and no difference in arthropod herbivore damage both conflict with the enemy release hypothesis, which posits that exotic species are successful because they suffer less damage from enemies relative to native species (Elton, 1958; Keane & Crawley, 2002). However, our results are consistent with evidence suggesting that exotic plants can suffer greater herbivory than natives (Parker, Burkepile, & Hay, 2006; Parker & Hay, 2005; Waller et al., 2020), supporting the biotic resistance hypothesis (Elton, 1958). Interestingly, broom, hares and nine of the eleven exotic plant species were all introduced from Europe to New Zealand, where they also regularly co-occur, suggesting that coevolutionary history in both their native and introduced ranges could contribute to the stronger direct and indirect impacts that we observed for the exotic than native plant species. Regardless of herbivore provenance, if exotic plants accumulate high densities of generalist herbivores and are more tolerant of this herbivory (Ashton & Lerdau, 2008), this may suggest high potential for strong indirect impacts on the surrounding community. Moreover, as the density of the invader and its interaction partners increases, we may expect both positive and negative indirect impacts to intensify as an invasion progresses. Therefore, we suggest that management practices targeting shared interaction partners as well as the focal invader may improve mitigation of negative invasion impacts or promotion of positive impacts. For example, in Otago, New Zealand, the invasive venomous redback spider *Latrodectus hasseltii* is facilitated by burrows of another invader, the European rabbit *Oryctolagus cuniculus*, and preys upon the critically endangered endemic Cromwell chafer beetle *Prodontria lewisii*. By filling in rabbit burrows, spider density and capture of the chafer beetle were decreased to zero (Spencer, van Heezik, Seddon, & Barratt, 2017).

Finally, in contrast to our expectations, we found that the strength of indirect impacts mediated by other interaction partners (i.e. soil fungi vs. hares) was generally a poor predictor of indirect impacts of broom (Table S5). This result was somewhat surprising in light of previous studies that have demonstrated that species interactions are sometimes correlated between mutualistic and antagonistic partners (Sauve et al., 2016). However, it may be that functional trait data specific to the species interactions investigated (e.g. plant defences, specific root length and root diameter) (Carmona, Lajeunesse, & Johnson, 2011; Eissenstat, Kucharski, Zadworny, Adams, & Koide, 2015) would better predict indirect interaction strength among species, especially when combined with phylogenetically informed quantitative interaction network analyses (e.g. Frost et al., 2016; Pearse & Altermatt, 2013; Tack, Gripenberg, & Roslin, 2011).

There are several limitations of our study that we would like to acknowledge. First, although we have simultaneously quantified

multiple direct and indirect impacts of broom on 21 native and exotic species, these were the impacts of a single invasive species at one site and over a single growing season. Second, all of the experimental species were legumes in order to minimize phylogenetic distance from broom and maximize their likelihood of sharing interaction partners, thereby limiting our ability to extend our findings to other plant families and meaning that inference outside of the family Fabaceae should be treated with caution. Moreover, this selection of closely related species could potentially generate bias towards the detection of strong indirect effects based on phylogenetic conservatism of species interactions (Chagnon, Bradley, & Klironomos, 2015; Peralta, 2016), although we found no evidence of this phylogenetic relationship within Fabaceae (Supporting Information S8, Figure S12). Thus, although these caveats may limit the inference space for invasions in general, we believe that our findings are at the forefront of scaling up experimental invasion ecology to the community level and present a framework for predictions that can be tested in other systems, while also providing critical local information on broom invasion and impacts in New Zealand.

We conclude that invader impacts occur through the balance of multiple direct and indirect pathways involving soil fungi, rhizobia and mammalian and arthropod herbivores. In our system, broom had contrasting impacts on native and exotic plant species above and below ground, but with a net advantage to exotics, suggesting that direct and indirect interactions may contribute to invasional meltdown of exotic plants. Furthermore, although the net outcome for species interacting with broom depends on both direct and indirect impacts, we found that indirect interactions acted through multiple mediating species and were over six times as strong as direct impacts, indicating that management practices should consider targeting shared interaction partners as well as the focal invader. Our results integrate multiple invasion hypotheses that invoke species interactions, and highlight the complex and unpredictable role of indirect interactions in driving invasion impacts on above and belowground communities as well as the importance of a community-level approach to studying biological invasions. Through taking a more holistic future perspective we will improve our understanding of the role biotic interactions play in the success of invaders and their impacts on natural communities.

ACKNOWLEDGEMENTS

The authors thank Brooksdale Station and Stu Gunn for field site access and to Hamish Maule for assisting with vegetation surveys and plant identification. We are also grateful to Jim Allen, Neroli Allen, Thomas Carlin, David Conder, Daniel Dash, Brian Kwan, Aimee McKinnon, Leona Meachen, Brent Richards, Georgia Steel and Angela Wakelin, for field and lab assistance, and to the ABiNZE, Weed Wing, Stouffer, Tylianakis and Ecosystem Mycology research groups for helpful discussions about the experiment. We also thank James Cronin, Ganesh Bhattarai and the anonymous reviewers for their feedback on earlier versions of the manuscript. This project was supported by Centre of Research Excellence funding to the Bio-Protection Research Centre from the Tertiary Education Commission and the Royal Society of New Zealand Hutton Fund. The authors declare no conflict of interest.

AUTHORS' CONTRIBUTIONS

I.A.D. and J.M.T. obtained funding; W.J.A., R.W., J.M.T., B.I.P.B. and I.A.D. designed the experiment; W.J.A., R.W., M.-R.S. and L.P.W. set up the experiment and collected data; W.J.A. conducted analyses and wrote the first draft of the manuscript, and all authors contributed substantially to revisions.

DATA AVAILABILITY STATEMENT

Data are available from the Dryad Digital Repository https://doi.org/10.5061/dryad.2v6wwpzjg (Allen et al., 2020).

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

How to cite this article: Allen WJ, Wainer R, Tylianakis JM, et al. Community-level direct and indirect impacts of an invasive plant favour exotic over native species. *J Ecol*. 2020;00:1–12. https://doi.org/10.1111/1365-2745.13433